

New Frontiers in Stress Research

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New Frontiers in Stress Research

Modulation of Brain Function

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PREFACE

A fundamental question in stress research today is when adaptive processes stop being essential for life and become damaging to health. Why some individuals are more vulnerable to chronic stress than others is also an unresolved phenomenon. These were two of the main issues discussed at the OHOLO Research Conference held on March 20–25, 1996 in Zich'ron Yakov, Israel.

The book offers a comprehensive overview of neural, endocrine and immunological responses to stress. We describe how these responses are generated and subsequently feedback and modulate brain function. The book presents an interesting mix of basic research papers and in-depth review papers. It combines experimental approaches in stress research from various disciplines ranging from clinical studies and behaviour to electrophysiology and molecular biology.

In Section I the organization of the stress response is discussed in its various modalities. These include the hypothalamic pituitary adrenal axis and the sympathetic nervous system, with emphasis on corticosteroid hormones, biogenic amines, neuropeptides and neurotrophic factors. Section II provides a view on the underlying mechanisms of stress-hormone action in the brain and how these mechanisms may be implicated in the damaging effects of long-term stress. Section III focuses on the development of the stress response system. Genotype and perinatal experience of the pup appear to program the stress response system for life, and the mother has a crucial effect on these organizational processes in the brain. Section IV focuses on neuro-immunological communication: it has become clear that cytokines released by immune cells modulate brain and behaviour. Section V addresses the implication of the stress effects in human conditions and emphasizes the search for pathogenetic mechanisms underlying Post Traumatic Stress Disorder (PTSD), depression and Alzheimer's Disease. The final contribution highlights the application of gene technology in the search for new drug targets in the treatment of stress-related disorders.

The Conference on which this book is based was held with the financial support from the Commission of the European Communities. The Editors thank the authors for their contributions. They hope that this book will be an invaluable source of information for basic researchers as well as for clinicians interested in the role of stress in vulnerability to the pathogenesis of stress-related brain disorders.

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I. Organization of the Stress Response

1 Corticosteroid Hormones and the Organization of the Stress Response System

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Every change in the internal or external environment that disturbs or threatens to disturb homeostasis evokes a spectrum of adaptive physiological responses. For instance, the sympatho-adrenomedullary response mediated by adrenaline causes an immediate rise in blood pressure and rapid mobilization of energy aimed to react immediately to a challenge. The hypothalamic-pituitary-adrenal (HPA) axis also mediates the animal's ability to cope with stress, but its hormones act strategically over a much longer time period. These neural, endocrine, and behavioural adaptations help to restore homeostasis.

Stress activates the HPA axis. This implies that secretory bursts of hypothalamic parvocellular corticotropin-releasing hormone (CRH) and vasopressin (VP), pituitary ACTH and adrenal corticosteroid hormones are increased in magnitude. The parvocellular neurons of the paraventricular hypothalamus (PVN) contain in addition to VP and CRH a great variety of other ACTH secretagogues. It has metaphorically been proposed that the pattern of these secretagogues released from CRH neurons is a signature representing the nature and intensity of the stressor (Romero and Sapolsky, 1996). Peak cortisol (man) or corticosterone (rodent) levels usually are attained 10–15 minutes after disturbance of homeostasis and the levels remain elevated for 30–90 minutes as a function of the nature of the stress and coping ability. Corticosteroid hormone action may persist for hours. The HPA axis and sympatho-adrenomedullary system act in concert and exert mutual control over each other's activity.

This chapter is on the central actions of corticosteroid hormone. The focus is on the action of corticosteroids in neural circuitry underlying the organization of the stress response, which is also critical for mood and mental performance. We will argue that: (1) the primary feedback site in regulation of the HPA axis is the CRH neuron in the paraventricular nucleus; Corticosteroids acting on hippocampus affect cognitive aspects of the stress response, which indirectly modify HPA regulation;

(2) aberrant corticosteroid levels and/or receptor deficits enhance vulnerability to stress-related disorders; and (3) such dysregulations in corticosteroid homeostasis are determined by genotype and early life events.

CORTICOSTEROID HORMONES AND THE STRESS RESPONSE SYSTEM

Some of the effects of cortisol (corticosterone) support the primary defense response of the organism to stressors. The hormone increases glucose stores and makes energy available during stress. It facilitates the action of other stress-response mediators (e.g., potentiation of the synthesis and action of adrenaline). These actions exerted by the hormone are "permissive" and generally involve transcriptional activation.

Glucocorticoids also form a second line of defense against stress by "suppression" of primary defense reactions. Inflammatory and immune responses are restrained and prevented from overshooting (Munck *et al.*, 1984). If corticosteroids fail to suppress, the primary defense reactions themselves become damaging and the organism becomes susceptible to inflammatory and autoimmune diseases. These suppressive actions of corticosteroids involve predominantly transrepression of stress-induced gene transcription (Table 1.1).

Corticosterone levels change due to circadian rhythmicity and in response to stress. The amplitude between peak and trough corticosterone levels may differ 50 to 100 fold. Yet under normal conditions, the mean steroid concentration over 24 hours is remarkably constant and represents the "set point" of corticosterone homeostasis (Dallman *et al.*, 1992). If corticosterone levels are too extreme, a centrally regulated "drive" modulates HPA activity in order to maintain its set point. Critical variables in the set point include: the properties of the corticosteroid receptors in brain and in pituitary corticotropes; the amount of biologically active free corticosterone available at the receptor site; and the responsiveness of the adrenal cortex to stimulation. The access of free corticosterone to its target sites depends on the local activity of steroid-metabolizing enzymes such as 11β hydroxysteroid dehydrogenase (11β HSD) Type 1 and Type 2, the properties of corticosteroid-binding globulin, and perhaps the activity of P-glycoproteins expressed by multiple drug resistance genes in endothelial cells (Van Haarst *et al.*, 1996; Rajan *et al.*, 1996; Funder *et al.*, 1988; Schinkel *et al.*, 1995; Hammond, 1990). These factors may amplify or dampen the corticosterone signal at the receptor site.

Table 1.1 Physiology and pathology of corticosteroid hormone action

<i>Physiology</i>	<i>Pathology</i>
Gluconeogenesis	Steroid diabetes
Suppressed growth	Catabolism
Increased cardiovascular response	Hypertension
Suppressed bone metabolism	Osteoporosis
Suppressed immune response	Immune deficit
Enhanced cognition	Impaired cognition

Pathological states of stress are often characterized by a change in set point of HPA activity. Thus, states of hyper- and hypocortisolemia develop as compensation to reach a new set point when the central "drive" to the HPA axis and the negative feedback action exerted by the steroids has become unbalanced. The change in set point can be deducted from the result of endocrine-challenge tests. For example, the escape (exaggerated by exogenous CRH administration) from dexamethasone suppression is thought to be indicative of a hyperactive stress response system. The feedback resistance revealed by this test is characteristic for depression with a reliability of more than 80% (Heuser *et al.*, 1994).

Chronically elevated corticosteroid levels cause such unwanted effects as breakdown of protein (catabolism), hyperglycemia (steroid diabetes), immune suppression, and susceptibility to infection, depression and steroid psychoses. Mental performance deteriorates and hippocampus volume reduces when circulating corticosteroid levels are chronically elevated (McEwen and Sapolsky, 1995). High corticosteroid concentrations enhance sensitization to stress- and drug-induced aminergic input in mesolimbic projection areas, causing increased vulnerability to drug-seeking behaviour by creating an addictive-prone state (Piazza *et al.*, 1991).

Syndromes characterized by hypoactivity of the stress system include posttraumatic stress disorder (Yehuda, 1996), chronic fatigue syndrome (Demitrack *et al.*, 1991), and primary fibromyalgia syndrome (Griep *et al.*, 1993). The lack of glucocorticoids produces pathological symptoms of myopathy, fatigue, hypotension, and susceptibility to autoimmune disorders and inflammation (Chrousos and Gold, 1992). Such hypoactivity may be of central origin or due to hyporesponsiveness of the adrenals to ACTH. The consequences of hypocorticism (other than its extreme form of adrenalectomy) for parameters in the central nervous system (CNS) have not been studied very extensively.

In summary, the various components of the HPA axis adjust their activity in order to maintain a set point in corticosterone homeostasis. Critical in set point regulation are the functioning of brain corticosteroid receptors and the adrenal output of corticosterone. Pathological states are characterized by changes in set point. As a consequence, the body and brain suffer from aberrant exposure to corticosteroid hormones.

TWO BRAIN CORTICOSTEROID RECEPTOR TYPES: DIFFERENTIAL EFFECTS

The mechanism of action for corticosterone in the brain relies on binding to high-affinity ($K_d=0.5$ nM) mineralocorticoid receptors (MR) and lower-affinity ($K_d=5$ nM) glucocorticoid receptors (GR) (Reul and De Kloet, 1985). Both receptor types belong to the superfamily of intracellularly located steroid receptors (Truss and Beato, 1993). The MRs bind aldosterone as well. The hippocampus lacks the inactivating enzyme 11β HSD type 2 isoform (Roland *et al.*, 1995; Rajan *et al.*, 1996; Van Haarst *et al.*, 1996). Therefore, corticosterone has, unlike in kidney, unlimited access to MRs in this brain region (see Seckl, this volume). Only in some defined periventricular regions 11β HSD is present and allows aldosterone-selectivity of the MRs (Roland *et al.*, 1995). These aldosterone selective MRs are thought to

be implicated in central control of cardiovascular functions, volume regulation, and salt homeostasis (Van den Berg *et al.*, 1994).

The difference in affinity implies that low circulating corticosterone levels occupy mainly the MR sites, while both (MR + GR) sites are occupied by high corticosterone levels following stress and during the circadian peak. GRs have a widespread distribution in neurons and glial cells, and their highest concentrations occur in key sites of brain stress circuitry, such as the limbic regions, the ascending aminergic neurons, and the CRH neurons. MRs have a much more restricted distribution and their highest densities are found in the neurons of hippocampus and septum (Table 1.2; Figure 1.1) (Van Eekelen *et al.*, 1988).

One of the main targets for corticosteroids in brain is the hippocampus. The neurons in hippocampal CA1, CA2, and dentate gyrus contain MRs and GRs, whereas CA3 expresses relatively much more MRs (Van Eekelen *et al.*, 1988). The spatial distribution of MR and GR was investigated in the CA1 neurons with double-labelling immunochemistry and confocal microscopy, combined with novel image restoration and image-analysis techniques. It was observed that hormone-activated MR and GR are localized in specific nuclear compartments in approximately 1000 clusters. Some clusters contain solely MRs or GRs, but a significant number contain both receptor types (Van Steensel *et al.*, 1996). It seems, therefore, that coordinated MR- and GR-mediated effects may occur in specific gene networks in nuclear domains that contain both receptor sites.

Using electrophysiology it appeared that ion regulation and transmitter responses of hippocampal neurons depend on a finely tuned balance of MR- and GR-mediated actions. In these cellular studies three important principles were uncovered for brain corticosteroid receptor function that also served as criteria for study of steroid action on other levels of biological organization (Joëls and De Kloet, 1994). First, the steroid actions mostly develop within 30–60 minutes and are *long-lasting*. They can be blocked with protein-synthesis inhibitors, suggesting a genomic mechanism of action. Second, the corticosteroids act *conditionally*, since the cellular effects are only detectable when the membrane is depolarized or hyperpolarized. Thus, the effects of the hormones may vary depending on the context of neuronal activation or inhibition. The molecular mechanism underlying these conditional corticosteroid effects is largely unknown. Third, the steroid effects display a *stringent receptor specificity*. For example, in CA1 neurons activation of MRs will stabilize excitability

Table 1.2 Properties of hippocampal corticosteroid receptor types

Receptor	Localization	Function
MR	Periventricular	Salt homeostasis
Aldo-selective	Hypothalamus	Cardiovascular regulation
MR		Behavioural reactivity
Cort-selective	Hippocampus	Sensitivity stress system
$K_d = 0.5 \text{ nM}$	Septum, amygdala	Stabilization neuronal activity
GR	Wide-spread in brain	Facilitation memory processes
$K_d = 5 \text{ nM}$		Recovery stress system
		Suppression stress-induced neuronal activity

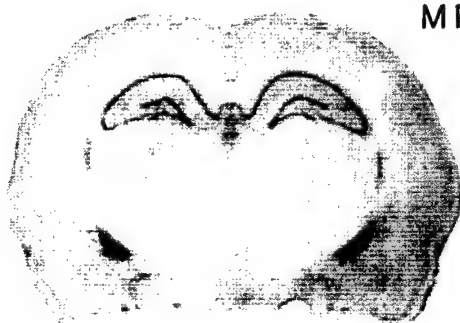
(3H)CORTICOSTERONE

MR



(3H)ALDOSTERONE

MR



(3H)RU28362

GR

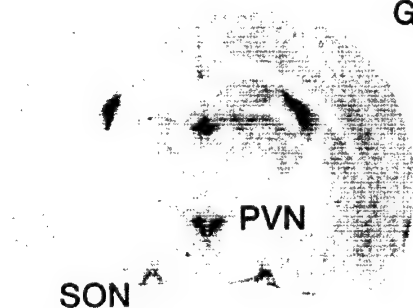


Figure 1.1 Localization of MR and GR in the brain. Tracer amounts of [^3H]-aldosterone, [^3H]-corticosterone and the pure glucocorticoid [^3H]-RU28362 were administered to adrenalectomized rats. One hour after administration the rats were killed and autoradiograms were generated. Note retention of aldosterone and corticosterone by hippocampal neurons, and of RU28362 by the PVN.

without allowing exposure of the cell to large amounts of Ca^{2+} ions or aminergic receptor stimulation. With the phasic GR occupation local excitability is reduced, due to suppressed responses to excitatory amino acid input and activation of Ca^{2+} -dependent K^{+} conductances (see Joëls, this volume).

These MR- and GR-mediated cellular events determine the function of hippocampal networks. Long term potentiation (LTP) and primed burst potentiation, depending on network function in hippocampus, are considered to represent a synaptic model for learning and memory processes. Interestingly, in its most optimal manifestation LTP depends on moderate steroid levels and predominant MR activation (see Diamond, this volume). This may contribute to the observation that, in intact animals, spatial learning is exceptionally sensitive to manipulation of the brain MRs and GRs (see Oitzl, this volume).

Using pharmacological approaches, the MR- and GR-mediated effects on behavior can be differentiated. A pulse intracerebroventricular (icv) administration of anti-mineralocorticoid reveals anxiolytic activity (Korte *et al.*, 1995) and behavioral reactivity is enhanced (Oitzl *et al.*, 1994). These responses suggest attenuated behavioral inhibition involving probably the hippocampal MRs (Gray and Rawlins, 1986). Blockade of stress-induced glucocorticoid occupation of brain GRs during a critical interval by pulse icv administration of the synthetic glucocorticoid antagonist RU 486 interferes with storage of learned information. The antiglucocorticoid also displays anxiolytic effects, which may be explained by the inability of the animal to store memory for fearful experiences (Korte *et al.*, 1995).

In summary, it is proposed that in hippocampal neurons colocalized MR and GR mediate differential effects of corticosterone in coordinated manners that are site specific, long lasting, and context dependent. These effects on neuronal function underlie cognitive processes associated with the stress response.

BRAIN CORTICOSTEROID RECEPTORS IN NEUROENDOCRINE REGULATION OF THE STRESS RESPONSE

Activation of the CRH neurons occurs with physical stressors such as infection, trauma, inflammation, respiratory distress, and hemorrhage. Cognitive stimuli are also particularly potent stressors under conditions of anxiety, uncertainty, lack of control, or poor predictability of upcoming events, either real or imagined (Levine *et al.*, 1978). The activation of the parvocellular CRH/VP neurons occurs through norepinephrine, serotonin and glutamate, while γ -aminobutyric acid (GABA) is inhibitory (Swanson, 1991; Herman *et al.*, 1995). Neuropeptides such as neurotensin, neuropeptide Y, opioids, substance P, and cytokines also modulate the activity of CRH neurons (Chrousos, 1995). In addition, the ascending inputs from locus coeruleus noradrenergic, raphe serotonergic and mesocortical dopaminergic neurons modulate the processing of information via action on limbic-forebrain circuitry including the hippocampus. This higher brain circuitry and the CRH neurons in the PVN are important targets for corticosteroid hormones (Figure 1.2).

Negative feedback action exerted by a stress-induced increase in corticosterone is mediated by GRs localized in the hypothalamic parvocellular CRH neurons, where

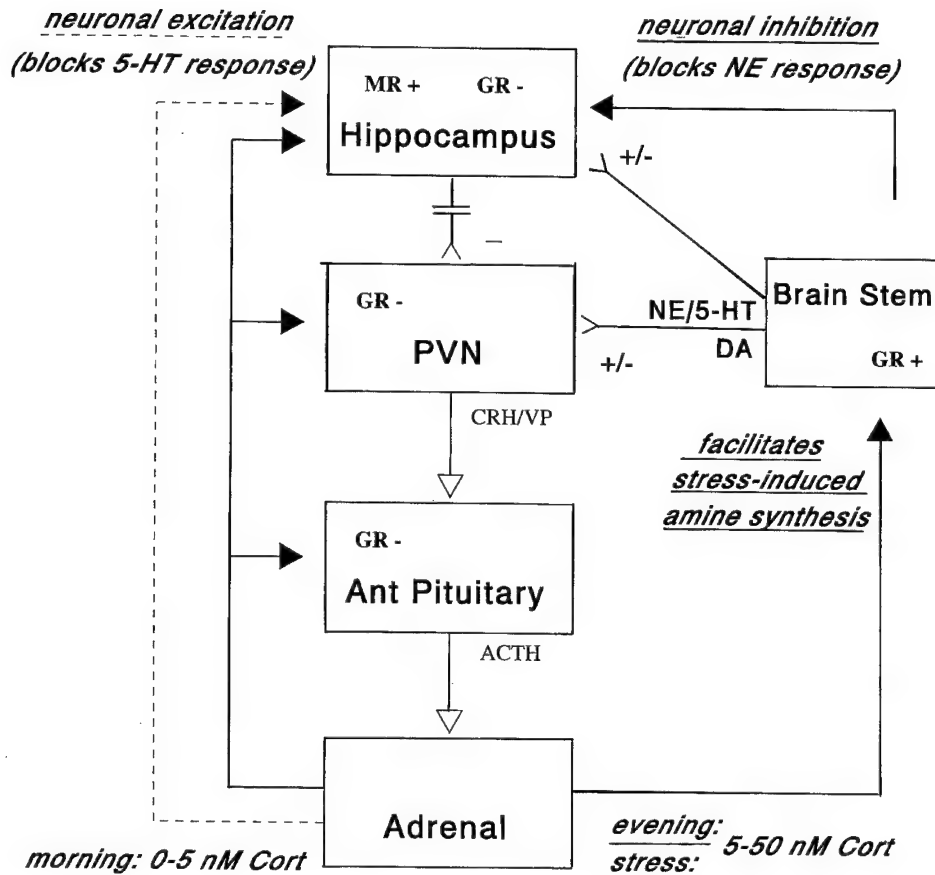


Figure 1.2 Schematic overview of MR- and GR-mediated effects in control of the HPA axis. Primary feedback site: corticosteroids suppress via GR in the paraventricular nucleus (PVN) stress-induced CRH and VP synthesis. GRs mediate stimulatory actions of corticosteroids on stress-induced aminergic input to the PVN. MRs in the hippocampus mediate an inhibitory influence. Episodic occupation of hippocampal GRs after stress or during the circadian peak is thought to act as a switch in disinhibition of negative MR-mediated control over the HPA-axis. The corticosterone concentrations indicate the free fraction of hormone.

stress-induced synthesis of CRH, VP, and other ACTH secretagogues is suppressed (Vamvakopoulos and Chrousos, 1994). The anterior pituitary corticotrophs are protected by a CBG-like molecule; only very high corticosterone concentrations suppress stress-induced ACTH release (De Kloet *et al.*, 1975). Paradoxically, feedback action of corticosterone outside the hypothalamus was found to promote stress-induced neural activation of parvocellular CRH. For example, the stress-induced activation of the CRH neurons is enhanced through GRs in the ascending aminergic neurons (Rots *et al.*, 1995).

It is important to realize that potent synthetic glucocorticoids such as dexamethasone act differently than the naturally occurring corticosterone. Systemic administration

of dexamethasone primarily has a pituitary site of action (De Kloet *et al.*, 1975). In fact, dexamethasone depletes endogenous corticosterone from the brain and is poorly retained by brain MRs. Accordingly, effects of corticosterone and dexamethasone on brain function and behavior are completely different, since the two steroids have opposite effects of MR/GR homeostasis (De Kloet, 1991).

Corticosteroids also exert through the hippocampus modulatory influences on HPA activity. Hippocampal output maintains transsynaptically an inhibitory GABA-ergic input to the CRH neurons (Swanson, 1991). Hippocampal MRs are prominent in the control of hippocampal inhibition over the CRH neurons under basal HPA activity conditions. First, pharmacological blockade with MR antagonists increased basal and stress-induced HPA activity (Ratka *et al.*, 1989). Second, in females the cyclic increase of HPA activity at the evening of pro-oestrus occurs when the high estrogen and progesterone levels impair MR function. Estradiol decreased MR mRNA and MR binding capacity in hippocampus and progesterone induced a significant decrease in affinity of MRs for corticosterone (Carey *et al.*, 1995). Third, rat strains with high hippocampal MR expression (e.g., Lewis rats) show lower HPA activity (Oitzl *et al.*, 1995). Fourth, aged individuals have generally reduced MR expression and disinhibited HPA activity (Van Eekelen *et al.*, 1991). Finally, tricyclic antidepressants increase hippocampal MRs and decrease HPA activity (Yau *et al.*, 1995; Holsboer and Barden, 1996).

The previous examples concerned physiological events, but in the pathophysiological realm interleukin-1 administration causes hypercorticism and sickness behaviour during the fever response, and reduces affinity of corticosterone to MRs in hippocampus. The impaired MR function is paralleled by a pronounced increase in the set point of HPA activity (Schöbitz *et al.*, 1994).

Antiglucocorticoids (e.g., RU 486 or mifepriston) applied near the PVN or icv enhance and prolong the stress-induced activity of the HPA axis (De Kloet *et al.*, 1988; Ratka *et al.*, 1989). As could be predicted from previous observations using agonist implants (Kovacs and Makara, 1988), GR antagonists injected in ng amounts in the hippocampus enhance stress-induced HPA activity (Van Haarst *et al.*, 1996). This observation is in agreement with electrophysiological studies that demonstrated MRs maintain or enhance a steady amino acid transmission in CA1 neurons, which is suppressed via GRs if transmission is transiently increased by stress (Joëls and De Kloet, 1994). Accordingly, brief exposures to high amounts of corticosterone disinhibit hippocampal influences on HPA activity. In contrast to previous views (Jacobson and Sapolsky, 1991), the hippocampal GRs therefore do not appear to mediate negative feedback of corticosterone on stress-induced HPA activity, but rather exert positive feedback through disinhibition.

In summary, these observations show that MR-controlled input from hippocampus to the CRH neurons is critical for the tone of the HPA activity. Hippocampal GR activation, as does GR activation in ascending aminergic neurons, produces opposite effects and counteracts the inhibitory control over CRF neurons. GRs in CRF neurons are the primary target of negative feedback action of corticosteroids on stress-induced HPA activity.

RESISTANCE TO GLUCOCORTICOID FEEDBACK

Aberrant levels of corticosterone develop if feedback resistance is acquired at the level of the CRH neurons. Such resistance becomes manifest if feedback control of stress-induced activation of CRH neurons fails, implying an imbalance between the activity of GR and membrane signalling pathways in these neurons. One way in which this balance can be disturbed is when a local GR deficit exists, as was recently demonstrated with genetically engineered transgenic mice (Barden *et al.*, 1995). Such mice display hypercorticism and signs of Cushing Syndrome. Resistance also can be achieved pharmacologically as was shown by chronic icv administration of GR antagonists.

Chronic blockade of GRs by infusion (100 ng/hour) of RU 486 in the lateral cerebroventricle did not affect basal a.m. levels of ACTH and corticosterone, but triggered sequential changes in HPA activity during the p.m. phase of the circadian cycle (Figure 1.3; Van Haarst *et al.*, 1996). Also the response to stress is enhanced and this facilitated ACTH response also occurs when inhibitors of corticosterone biosynthesis are applied (Dallman *et al.*, 1992). Ultimately, after 4 days of icv anti-glucocorticoid administration, the experimentally evoked central glucocorticoid resistance led to increased peak corticosterone levels during the p.m. phase. Increased weight and responsiveness of the adrenals to ACTH was also observed (Van Haarst *et al.*, 1996). Cognition actually was improved upon chronic icv RU 486 (Oitzl, this volume) as judged from performance of the rats in the Morris water maze. At the same time MR-mediated functions involving search strategies in the maze were enhanced, underscoring that the MR-mediated events become prominent upon blockade of GRs.

While chronic antiglucocorticoid enhanced the amplitude in circadian changes of corticosterone, rats implanted sc with slow-release corticosterone pellets (35–40% corticosterone) displayed flattened circadian rhythm (Akana *et al.*, 1992). While within physiological limits, chronic corticosterone suppressed the peak, increased trough levels, and obviously eliminated phasic changes in MR and GR occupancy. As is the case in chronic antiglucocorticoid treatment, chronic corticosterone altered the amplitude and the phasic changes in receptor occupancy, but not the 24-hour adrenocortical output nor the set point of HPA activity. These changes were associated with an attenuation of the HPA response to stress. In a recent study it was convincingly demonstrated that chronic corticosterone administration did not cause symptoms of hypercorticism such as thymus involution. Instead such animals with a flattened corticosterone rhythm appeared an excellent animal model to study MR-mediated effects on serotonergic function in hippocampus (Meijer *et al.*, submitted). However, if chronic corticosterone substitution actually increases the amount of circulating steroid over set point levels, all signs of hypercorticism and chronic stress appear, including worsening of cognition (Luine *et al.*, 1994).

Reset of feedback sensitivity most commonly is acquired during exposure to chronic stress. Such steroid resistance may be due to transcription factors activated by membrane receptor-coupled signalling. AP1, CREB and NF κ B are activated via membrane signalling in response to stress, inflammation, and immune stimulation. GRs repress the generally positive effects by these transcription factors, either

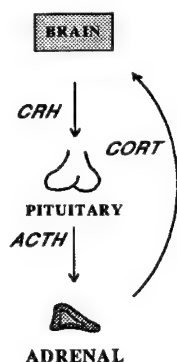
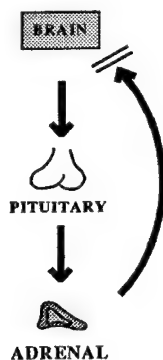
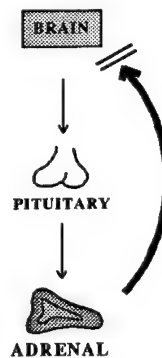
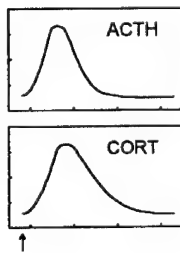
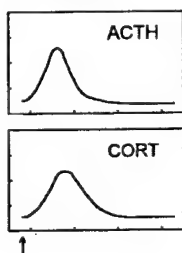
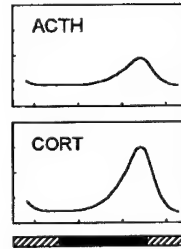
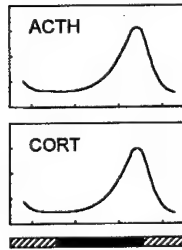
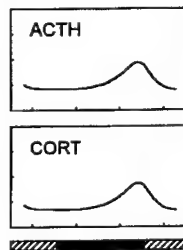
NORMAL**ACUTE****CHRONIC****Stress****Circadian rhythm**

Figure 1.3 Schematic representation of sequential changes following pharmacological induction of glucocorticoid resistance. From left to right are represented: the normal situation, the acute changes after central GR blockade, and the effects of chronic blockade of central GRs. Initially, i.c.v. infusion of glucocorticoid antagonist enhances the activity of the pituitary and adrenals during the circadian surge and in response to stress. At 3 days after chronic infusion of antiglucocorticoid adrenal hypertrophy as well as elevated steroid secretion at the time of the circadian peak have developed, whereas ACTH levels are normalized.

directly by composite GREs or by protein-protein interaction (Vamvakopoulos and Chrousos, 1994). Inadequate repression by GRs in this crosstalk of signalling pathways is characteristic for steroid resistance. Chronic stress possibly may cause alternate splicing of the GR gene. Recently a GR β variant of the common GR α receptor was found. GR β does not bind glucocorticoids, but inhibits transactivation by the α form (Bamberger *et al.*, 1996). However, GR β still needs to be demonstrated in CRH neurons.

Glucocorticoid resistance acquired in CRH neurons causes increased HPA activity and as a consequence the rest of the body, including the stress response circuitry in the brain, suffers from glucocorticoid *overexposure*. In contrast, if feedback supersensitivity has developed, then underexposure to glucocorticoids is the consequence. The hippocampus is extremely vulnerable to changes in corticosteroid signalling. This enhanced vulnerability is in part due to steroid-induced modulation of peripheral energy metabolism and immune responses. However, direct effects on integrity of hippocampal circuitry showing site specificity and regional differentiation also have been recognized. Thus, chronic hypercorticism causes atrophy of apical dendrites in the CA3 pyramidal neurons (Gould, 1994; McEwen, 1994) and enhances the vulnerability of CA1 neurons to excitotoxic and metabolic challenges (Sapolsky, 1992, 1996). In contrast, lack of corticosteroids causes cell death in the dentate gyrus through apoptosis (Sloviter *et al.*, 1993). It remains to be demonstrated how these differential and site-specific effects in the hippocampal circuitry relate to the U-shaped dose-response characteristics of neurophysiological responses on the one hand (Joëls and De Kloet, 1994; De Kloet and Joëls, 1996) and the syndromes of hypo- and hyperactivity of the stress system on the other (Chrousos and Gold, 1992).

In summary, feedback resistance or supersensitivity may be acquired in the CRH neurons. The underlying mechanism of these changes is set point regulation of the HPA axis and is largely unknown. However, adaptive changes in circulating corticosterone levels occur, which result in chronic over- or underexposure of extrahypothalamic regions to corticosterone.

CORTICOSTEROID-SEROTONIN CROSS TALK: RELEVANCE FOR PATHOGENESIS OF DEPRESSION

One system that is physiologically dependent on corticosteroids and sensitive to deleterious effects of hypercorticism is the brain serotonin (5-HT) system. This relationship may be significant for depression, as this disorder is often associated with hypercorticism, and drugs that specifically enhance serotonergic neurotransmission are effective antidepressants. Corticosterone affects many aspects of the 5-HT system: availability of tryptophan (the precursor of 5-HT); regulation of the activity of tryptophan hydroxylase (the rate-limiting enzyme of 5-HT synthesis); 5-HT release; expression levels of 5-HT receptors; and the cellular response to 5-HT receptor activation (Chaouloff, 1995). The effects of corticosterone are particularly clear for the 5-HT_{1A} receptor-mediated transmission in the hippocampus.

First, expression levels of the 5-HT_{1A} receptor mRNA and protein are suppressed by corticosterone in distinct subfields of the hippocampus of rodents (Meijer and

De Kloet, 1995; Meijer *et al.*, 1996). This case of gene suppression is sensitive to activation of MR, which makes the level of 5-HT_{1A} receptor expression in the hippocampus a function of basal corticosterone levels. It was recently shown that intact rats with an attenuated daily corticosterone profile (see above) had suppressed levels of 5-HT_{1A} receptor expression in the dentate gyrus of the hippocampus, and that this effect of elevated corticosterone was a consequence of excess MR activation at the time of the diurnal trough (Meijer *et al.*, submitted).

Second, the response to 5-HT_{1A} receptor activation of individual CA1 pyramidal neurons *in vitro* depends on the relative activation of MR and GR. Predominant activation of MRs will lead to an attenuation of the (hyperpolarization) response to activation of 5-HT_{1A} receptors. This suppression of the 5-HT_{1A} receptor-mediated response is independent of (and develops much faster than) the effect of corticosterone on 5-HT_{1A} receptor mRNA levels. High levels of corticosterone restore and enhance responsiveness to 5-HT_{1A} receptor activation through activation of GRs (Joëls and De Kloet, 1994).

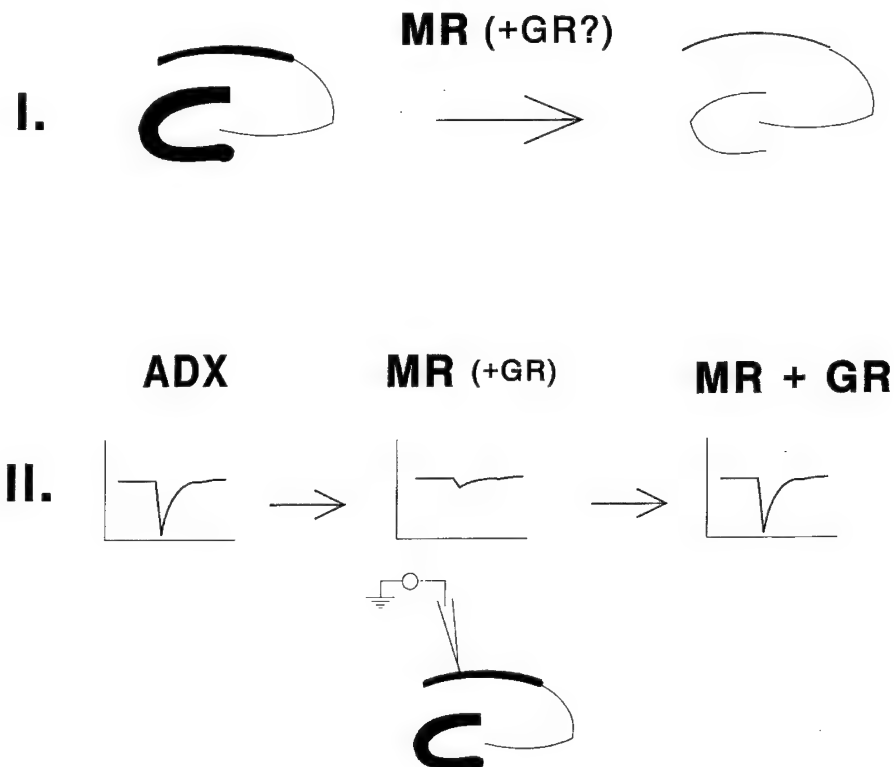


Figure 1.4 Corticosteroid control of midbrain serotonergic input in the hippocampal. (I) Corticosterone tonically suppresses the expression of 5-HT_{1A} receptor mRNA in distinct hippocampal cell fields via MR and possibly GR activation. (II) The electrophysiological response of CA1 pyramidal cells *in vitro* is differentially regulated by corticosterone. Predominant MR occupation leads to suppression of the hyperpolarizing response to activation of the 5-HT_{1A} receptor; combined (MR + GR) occupation restores the responsiveness to 5-HT_{1A} receptor stimulation.

Transiently elevated corticosterone levels are stimulatory to 5-HT transmission in the hippocampus and other brain areas via activation of GRs, while MR activation suppresses 5-HT_{1A} receptor-mediated hippocampal transmission (Figure 1.4). *Chronically* elevated corticosterone levels impair serotonergic transmission, but it is unclear through which mechanisms (Beck *et al.*, 1996). It may be that chronic activation of both MR and GR leads to downregulation of factors involved in the transduction of serotonergic signalling in neurons. Another possibility is that chronic severe hypercorticism induces a local GR resistance, which could lead to a lack of stimulatory GR-mediated effect and exaggerated suppressive MR-mediated effects (Meijer and De Kloet, 1996).

In summary, serotonergic neurotransmission in the hippocampus is dependent on the circulating levels of corticosterone. Basal levels of corticosterone exert a tonic suppressive effect (MR), while transiently elevated levels are stimulatory (MR + GR). In disease states, chronic hypercorticism occurs in parallel to impaired functioning of the serotonergic system.

NEONATAL ORGANIZATION OF THE STRESS SYSTEM IN THE RAT

The set point of HPA activity depends on genotype, the life history of an individual, and stress-coping ability. In particular, early life experience has profound and lasting effects on adult HPA set point. The features of the developing HPA axis are described below with a particular emphasis on the role of maternal-pup interaction.

HPA Development

During the first two postnatal weeks, corticosterone, ACTH, and hypothalamic CRH levels remain at very low constant levels. This period, which extends in the rat from day 4 until day 14, is further characterized by the fact that stressors which during adulthood elicit a vigorous HPA response are only weakly active in the infant. Therefore, this first two weeks of life are termed the "stress hyporesponsive period (SHRP)." Further development of the HPA axis does not proceed uniformly; the various components have different ontogenetic patterns. Thus, while the stress response is present at day 15, circadian rhythmicity and feedback regulation reach adult levels only after 4 weeks (Rosenfeld *et al.*, 1992).

The corticosterone levels during the SHRP are very low, but the hormone circulates in the unbound form, since during this period CBG levels are not detectable. Corticosteroid receptor types are present in brain (e.g., in the pituitary) and have distinct developmental patterns. GR levels gradually increase from the low initial levels to adult levels by the fourth week of life. GR microdistribution changes dramatically during development. For example, the suprachiasmatic nucleus expresses high levels of GR only during the first week of life (Van Eekelen *et al.*, 1991). MRs are present in very low concentrations the first day of life, whereupon the concentration rapidly rises, and resembles that found in adulthood at the end of the first week (Rosenfeld *et al.*, 1992). In view of the low corticosteroid levels it is

likely that most of the physiological actions of the hormone during SHRP are exerted via the MR site involved in stabilization of the stress response system.

The major rate-limiting factors in HPA activation during the SHRP are at the level of the brain and the adrenals (Figure 1.5). At the brain level, immaturity of stimulatory neural pathways to the hypothalamic CRH/AVP neurons is responsible. The various pathways have a variable ontogenetic development, which may explain why a painful stimulus triggers a CRH response at earlier postnatal days than does exposure to novelty. The aminergic pathways are only fully developed at postnatal day 30. The adrenals show reduced sensitivity to ACTH (Rosenfeld *et al.*, 1992).

HPA-AXIS DURING DEVELOPMENT

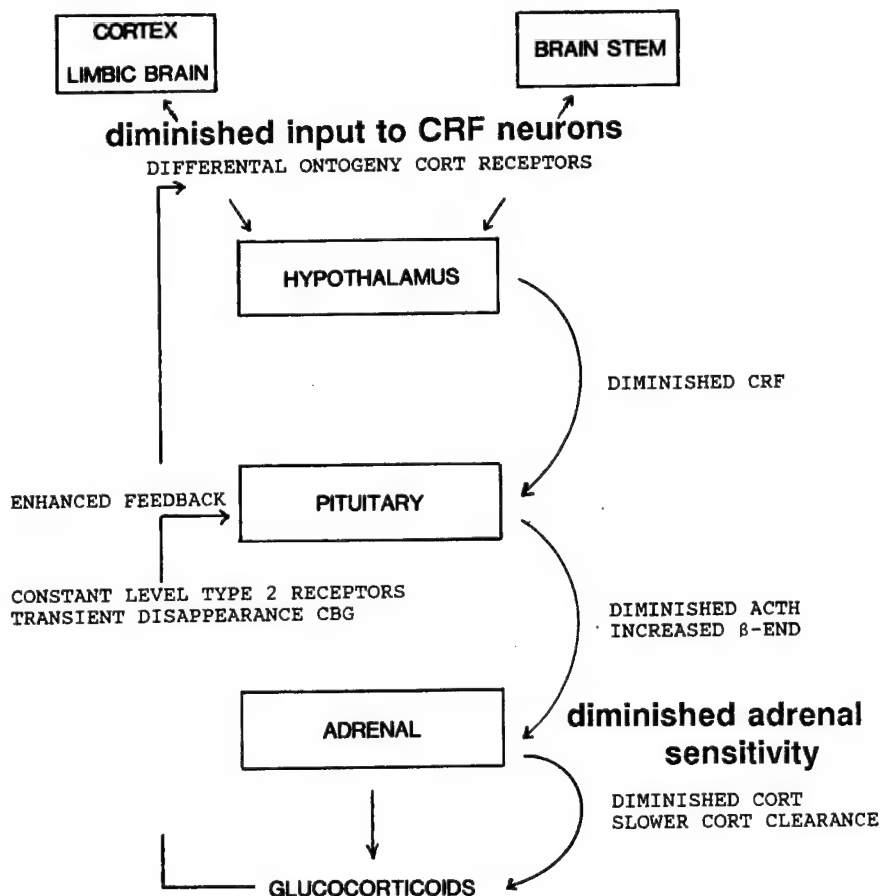


Figure 1.5 HPA axis during development. Diminished input to the CRH neurons and diminished adrenal sensitivity are the major causes of the hyporesponsive period in the corticosteroid hormone response to stress during postnatal day 4 to 14 in the rat.

Enhanced negative feedback of corticosterone has been postulated as causal for SHRP. This can be excluded, however, since the magnitude of the feedback signal is not greater than observed in the adult animals. Thus, constant low levels of corticosteroids are indispensable for normal maturation and critical for neurotransmitter phenotype. For example, without GR (in mice homozygous for GR gene disruption) the sympatho-adrenomedullary system was poorly developed (Cole *et al.*, 1995). Low levels facilitate differentiation of a previously established phenotype (e.g., increased tyrosine hydroxylase [TH] in sympathetic neurons and phenyl-N-methyl transferase [PNMT] in chromaffin cells; Rosenfeld *et al.*, 1992).

Maternal Regulation of the Infant HPA Axis

Studies using maternal separation have demonstrated that the mother regulates physiological responses in the infant. These physiological responses slowly develop as a function of time after maternal separation and appear tightly linked to specific aspects of mother-infant interaction. For example, if pups are separated from the mother for 24 hours there is a 40% decline in heart rate. This decrease results specifically from the absence of milk, as opposed to the lack of maternal contact or other aspects of maternal care, as normal infant cardiac rate is resumed following milk infusion. Growth hormone secretion is also reduced after maternal separation. However, this decline appears due to the lack of maternal tactile stimulation, rather than milk deprivation. Vigorous stroking of the deprived pup with a moistened brush normalizes growth hormone secretion. Sleep/wake cycles are also disturbed after maternal separation, and this disturbance is corrected by suckling rather than feeding *per se* (Levine, 1994).

While cardiac response, growth hormone secretion, and sleep/wake rhythm appear all under the stimulatory influence of maternal care, the presence of the mother suppresses infant HPA activity. Maternal deprivation for 24 hours causes small but significant increases in corticosteroid levels over the course of the separation. Depending on the infant's age and duration of separation, the effects on HPA activity may be greater. Thus, separation of mother and infant for at least 8 hours up to 24 hours at day 3 is needed to sensitize the adrenals the next day for response to exogenous ACTH or to the stress of an ip saline injection. Responses to ether, novelty, and saline injection immediately following deprivation are larger at 9, 12 and 16 days of age, than at day 4 (Levine, 1994). To further complicate the issue, the day of separation during the SHRP is also important. The ACTH response to saline or ether measured at day 20 is enhanced if the animal is deprived for 24 hours at day 3, but suppressed if the same procedure was applied at day 12 (Van Oers *et al.*, submitted; Workel *et al.*, submitted).

Permanent effects of maternal deprivation have been found on corticosteroid receptor levels in brain. Hippocampal GR number is reduced in 2-month-old male adult Wistar rats deprived for 24 hours at day 3 (Sutanto *et al.*, 1996). The male rats had increased adrenal weight and circulating basal ACTH and corticosterone. These rats displayed increased apomorphine-induced stereotypical gnawing, which is an index for increased responsiveness of the nigrostriatal dopaminergic pathway (Rots *et al.*, 1996). In contrast, in the same experiment maternally deprived female rats showed as adults an increased Bmax of hippocampal GR (Sutanto *et al.*, 1996).

Maternal behaviour ensures during development a quiescent stress response system in the newborn rat, which is characterized by low and constant corticosterone levels. Maternal deprivation of the infant for 24 hours disrupts the SHRP. As a result inappropriately high levels of corticosterone during the deprivation stress are thought to interfere with normal brain development (Levine, 1994). A similar outcome is observed if pups are deprived for 3 hours per day from the mother for the first 2 postnatal weeks (Plotsky and Meaney, 1993).

The "handling" procedure is only a brief separation of pups from the mother for 15 minutes, once per day, until the age of weaning. Thus, handling provides a brief intermezzo in mother-pup interaction, which subsequently results in increased sensory stimulation by intensified maternal care triggering a set of physiological responses. The HPA activity remains low and stable during handling. According to the working model by Meaney and colleagues (1988), increased thyroid hormone enhances 5HT₂ receptor stimulation in hippocampus, which then somehow leads to an increase in GR gene expression.

Handling enhances maturation of the HPA circadian rhythm and the glucocorticoid negative feedback response, and thus achieves a reduction in duration of the SHRP. As adults, the neonatally handled animals exhibited reduced fearfulness, since the animals explored a novel environment, in contrast to the unhandled animals that crouched in a corner. Neonatally handled rats showed, as adults, more rapid activation and termination of the adrenocortical responses to stress than their unhandled littermates. This apparently enhanced inhibition of stress-induced HPA activity seems associated with increased hippocampal and frontal cortex GR number. During senescence, handled animals displayed better performance in a Morris maze test designed to evaluate cognitive functions of the rat.

In summary, during the first two weeks of life the stress response system is hyporesponsive resulting in low and constant corticosterone levels. Development of the stress system can, however, be permanently altered if pups are separated from the mother. As adults the outcome for emotion, cognition, and adrenocortical reactivity depends on the strain, sex, and the age of the pup, as well as the duration and frequency of the separation procedure.

CONCLUDING REMARKS

Numerous neurologic and psychiatric disorders have a genetic background, which is in most cases multifactorial. The onset and progression of the disorders in predisposed individuals is in part also dependent on environmental and experience-related factors. In this chapter we have argued that these factors activate neural signalling pathways, which in crosstalk with corticosteroid hormones and via their two main types of brain receptors control the expression of these "disease" genes. The brain receptors mediate distinct and opposite actions of corticosterone, which in a coordinated manner control neuronal circuit function. The fact that the corticosteroid action is site-specific, long-lasting, and conditional implies their nuclear receptors as a target for a new lead in treatment of stress-related brain disorders.

Acknowledgement

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2 The Role and Regulation of Monoamines in Stress

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STRESS RESPONSE SYSTEMS

In biology the term “stress” is generally used to refer to the bodily response that occurs in the presence of challenges to psychological and/or physiological homeostasis. Stress acts as a warning signal, generated in the brain, detecting danger in the environment and indicating that action is required immediately (Gray, 1987). Only when this regulatory mechanism fails to function properly and promptly do abnormal pathological conditions (e.g. anxiety disorders or depression) interfere with healthy well-being. The hallmarks of the stress reaction are activation of the sympathetic-adrenal medullary (SAM) system and the hypothalamic-pituitary-adrenal (HPA) axis. The monoamines play an important role in both systems. It is the purpose of this chapter to elucidate these roles and speculate on how levels of the monoamines may be regulated.

The catecholamines (CAs) noradrenaline (NA) and adrenaline (A) plus the indoleamine serotonin (5-HT) are the major monoamines discussed in this chapter. Plasma CA levels have been shown to be elevated by acute stressors and the degree of increase reflects the intensity of the stressful stimuli (Natelson *et al.*, 1981; Goldstein *et al.*, 1983). It has been suggested that changing levels of plasma NA during stress primarily reflect sympathetic nervous outflow whereas plasma A levels derive from adrenal medullary secretion (Axelrod and Reisine, 1984). Activation of the HPA axis, via increased corticotrophin releasing factor (CRF) production in the hypothalamus, increases release of adrenocorticotrophin hormone (ACTH) from the anterior pituitary, which triggers glucocorticoid secretion (cortisol, corticosterone, and cortisone, of which cortisol accounts for about 95% of the total in humans) from the adrenal cortex (Mason *et al.*, 1968). Plasma, urinary and salivary cortisol levels have been the most widely used peripheral marker of physiological stress responses (e.g. Kirschbaum *et al.*, 1993).

Even though these systems are often studied in isolation, there is known to be interaction between the SAM and HPA systems. During stress the adrenal medulla of mammals is directly exposed to high levels of cortical glucocorticoids which activate the medullary enzyme phenylethanolamine-N-methyltransferase (PNMT). Thus stress-induced activation of the HPA axis, or intervention by ACTH injection, influences the SAM system by elevating PNMT activity with a concomitant increased conversion of NA to A (Hucklebridge *et al.*, 1981). Furthermore there is evidence that adrenal-cortex-derived glucocorticoids dampen sympathetic neuronal activity under basal conditions and during stressful stimulation (Kvetnansky *et al.*, 1993). Conversely, free plasma monoamines can stimulate ACTH release through a direct pituitary mechanism (Axelrod and Reisine, 1984; Dinan, 1996) and glucocorticoid release directly from the adrenal cortex (Dinan, 1996).

Monoamines also play a role in the regulation of the HPA axis in the brain (see Dunn and Kramarcy, 1984). Activation of the paraventricular nucleus (PVN) of the hypothalamus produces CRF and is the key step in initiating the HPA response. The question of how the PVN itself is regulated therefore becomes a vital issue in the understanding of stress responses. Just as there is no single stimulus that represents a threat to homeostasis, there is no single brain pathway projecting to the PVN responsible for its activation. Many neurotransmitter pathways project to the PVN and doubtless contribute to its functioning to varying degrees but the main NAergic projection from the locus coeruleus is important (Plotsky *et al.*, 1989). Lesions of this pathway, although not affecting basal CRF levels, do abolish the hypothalamic response to acute (Harbuz *et al.*, 1991) and chronic stress (Harbuz *et al.*, 1994). Similarly, serotonergic projections from the raphe nuclei to the PVN have been shown to be important in the regulation of CRF (see Dinan, 1996).

Brain monoamine neurotransmitters also play a key role in mood and behavioural control. A functional excess of monoamines (particularly NA and 5-HT) is associated with mania and anxiety, whereas a deficiency is associated with depression (the monoamine hypothesis of affective illness, see Schildkraut, 1965). In this way monoamines serve as the intermediary between affective processes and sensitivity to the stress response.

REGULATION OF THE MONOAMINES

These complex interrelationships between the SAM and HPA systems, both involving the monoamines, reveal the subtleties of the stress response. Ultimately, in both systems, the levels of available monoamines can influence this response such that the greater the availability of monoamines, the greater the stress response. This leads us to examine the factors that control monoamine availability. Monoamine levels are determined by a combination of factors including precursor availability (especially important for 5-HT) and rate-limiting synthetic enzymes (e.g., tyrosine hydroxylase, which is especially important for NA). Adrenal medullary tyrosine hydroxylase activity is facilitated by activity in the sympathetic preganglionic neurons, which maximizes availability of NA during the SAM stress response (Bhagat and Horenstein, 1976). Endogenous regulation of the metabolism of

monoamines by the enzyme monoamine oxidase (MAO) is important for ALL monoamines and is the focus of the rest of this chapter.

Monoamine Oxidase

Since its discovery almost 70 years ago (Hare, 1928; Blaschko, 1974), MAO has been found to be widely distributed in the animal kingdom (Hall and Uruena, 1983). It exists in two distinct forms, MAO-A and MAO-B (Johnston, 1968), which differ in substrate and inhibitor specificities: the preferential substrates for MAO-A are NA and 5-HT, whereas MAO-B acts preferentially on unsubstituted aromatic amines such as phenylethylamine (PEA) and benzylamine. Dopamine and tyramine are nonselective substrates for both MAO-A and MAO-B (see Denney and Denney, 1985). Both forms of the enzyme are integral proteins of the outer mitochondrial membrane (Schnaitman *et al.*, 1967). Many tissues, such as human liver and brain, express both MAO-A and MAO-B (Murphy and Donnelly, 1974), whereas others primarily express one form. For example, placental trophoblasts express MAO-A (Egashira, 1976) whereas platelets (Donnelly and Murphy, 1977) and lymphocytes (Bond and Cundall, 1977) express only MAO-B.

Rapid changes in MAO activity are possible, having been reported in relation to circadian rhythms in the hypothalamus (Follet, 1969; Urry and Ellis, 1975). Even small fluctuations in MAO activity can induce changes in monoamine levels that have functional significance (see Murphy, 1984). Hence, MAO-A inhibitory drugs (e.g., phenelzine and iproniazid), which facilitate NA and 5-HT neurotransmission, have been found useful for the treatment of depression (Ad Sitsen and Montgomery, 1994). (This treatment can cause hypertensive attacks when associated with the consumption of food rich in tyramine, an indirectly acting sympathomimetic amine [Youdim and Finberg, 1982].) Indeed, it has been proposed that an abnormality in MAO regulation may even be the cause of depression (Murphy, 1984). Earlier Welch and Welch (1970), after observing the levels of brain monoamine metabolites produced during different experimental stress conditions, had suggested that the brain monoamine response to acute stress could best be explained by a rapid natural inhibition of MAO. Consistent with this hypothesis, Maura and Vaccari (1975) demonstrated that environmental stress led to a significant decrease in rat liver and brain MAO activity, but that the activity returned to normal after 7 days. Thus, indirect evidence suggested the existence of an endogenous inhibitor(s) of MAO that play a key role in the modulation of monoamine levels and was important in the stress response.

ENDOGENOUS INHIBITION OF MONOAMINE OXIDASE

Human Clinical Studies

The first direct evidence that this may indeed be the case came in 1980. Extracts of human urine were shown to inhibit competitively MAO (Glover *et al.*, 1980). Urine samples from 14 normal subjects gave varying degrees of inhibition when assayed

using rat liver as the enzyme source. When assayed using human platelet MAO, the same samples gave similar results, demonstrating that the inhibitor was not generated by the rat liver. The inhibitory activity was found to be competitive and due to a low molecular weight fraction (approximately 200 Da) that extracted into ethyl acetate at pH 1, implying it was either neutral or acidic. The samples inhibited both MAO-A and MAO-B. Various known urinary constituents (e.g., urea, uric acid, creatinine, glycine, L-histidine, hippuric acid, citric acid, formic acid, ammonia, Na, Cl, Ca, K, Mg and P) were assayed in isolation and combination for their effect on MAO activity. None were found to be responsible for the inhibitory activity found in urine. Monoamines, their metabolites, and their conjugates were also assayed with no significant inhibitory activity noted. This endogenous MAO inhibitory activity (MAO-I) was named 'tribulin' in 1982 (Sandler, 1982).

Soon after its discovery, tribulin activity was determined in 24 hour urine samples from 50 psychiatric patients and 28 control subjects. Postwithdrawal alcoholics showed significantly higher MAO-I than controls, whereas depressed or schizophrenic patients showed slightly lower MAO-I than controls. When the patients were split into two groups based on their symptomology, the patients with agitated depression, mania, acute schizophrenia, and postwithdrawal alcoholics showed significantly more MAO-I than retarded depressives and chronic schizophrenics. This led to the conclusion that increased urinary MAO-I was probably not pathology specific, but a marker for increased stress, anxiety and arousal, conditions common to the first group of patients (Petursson *et al.*, 1981). This study was followed by a report of increased urinary MAO-I in patients withdrawn from benzodiazepines. Urinary MAO-I activity was found to correlate with anxiety as assessed by the Hamilton Anxiety questionnaire and was independent of urinary volume, creatinine concentration, or pH (Petursson *et al.*, 1982). High urinary MAO-I was again reported in postwithdrawal alcoholics compared to controls (Bhattacharya *et al.*, 1982).

Further human studies continued to reveal significant correlations between urinary tribulin output and feelings of increased stress and anxiety. The stress of maximal exercise was found to increase both plasma and urinary NA as well as urinary MAO-I (Armando *et al.*, 1984). In a study of neurological patients (21 epileptics and 12 other neurological inpatients), 24 hour urine samples showed that all patients had significantly higher urinary MAO-I compared with controls (Clow *et al.*, 1987). The authors concluded that increased tribulin output may be common to all neurological disorders and that the increase may be related to the stress and anxiety of the patients. Following this work investigations into patients with panic disorder (between attacks) revealed no difference between urinary MAO-I of this group compared with controls (Norman *et al.*, 1988). In the same year Clow *et al.* (1988a) measured urinary output of MAO-I, vanillylmandelic acid (VMA), homovanillic acid (HVA), and 3-methoxy-4-hydroxyphenylglycol (MHPG) during lactate-induced panic attacks in patients with agoraphobia and panic disorder. They found that urinary MAO-I increased significantly while the concentrations of the catecholamine metabolites VMA and HVA were significantly decreased compared with controls. A nonsignificant decrease in MHPG (the predominant CNS catecholamine metabolite) was found. Although this study did not measure the output of parent monoamines, other studies (not measuring tribulin activity) have

reported increased plasma monoamine levels during panic attacks (Liebowitz *et al.*, 1984; Carr *et al.*, 1986). These results suggested that the rise in endogenous MAO-I results in decreased output of monoamine metabolites and increased endogenous monoamines (Clow *et al.*, 1988a). This report also supported the suggestion by Norman *et al.* (1988) that tribulin is a state-anxiety marker. A follow-up study investigating a group of patients with general anxiety disorder revealed increased urinary MAO-I in this group compared to controls, again supporting the argument that tribulin activity is increased in anxiety (Clow *et al.*, 1988b). In contrast, a group of patients with posttraumatic stress disorder (PTSD) did not appear to have increased urinary MAO-I compared to controls (Davidson *et al.*, 1988). However, the patients were not experiencing an acute episode of PTSD, and so were not in a stressed state, which may explain this finding. Finally, migraine sufferers had a significant increase in urinary MAO-I at onset, during, and after an attack compared to controls and migraine-free periods. This again indicated that tribulin output is raised during stressful periods (Jarman *et al.*, 1991).

Animal Studies

Cold-restraint stress has been shown to induce a fivefold increase in rat urinary MAO-I (Glover *et al.*, 1981). This increase was independent of urinary creatinine and pH and could be attenuated by the administration of lorazepam prior to the stressor. A range of studies went on to demonstrate that various stressors could elicit increases in tribulin activity in rats: cold-restraint stress increased urinary (Glover *et al.*, 1981), heart (Armando *et al.*, 1988), and brain (Bhattacharya *et al.*, 1988) MAO-I; repeated isolation stress increased MAO-I in brain, cerebellum, heart, and kidney (Armando *et al.*, 1989); isolation increased MAO-I in brain and kidney (Hamaue *et al.*, 1992); and footshock increased MAO-I in both heart and brain (Lemoine *et al.*, 1990; Lemoine *et al.*, 1994). Increased anxiety and brain MAO-AI and MAO-BI was found when rats were withdrawn from morphine, ethanol, lorazepam, or nicotine with no differences in anxiety, MAO-AI, or MAO-BI in animals withdrawn from cannabis extract or ondansetron (Bhattacharya *et al.*, 1995). Pharmacological induction of anxiety by administration of pentylenetetrazole to rabbits has also been shown to increase brain (but not liver) MAO-I (Clow *et al.*, 1989a). Similarly pentylenetetrazole (and yohimbine) caused increased rat brain MAO-I (Bhattacharya *et al.*, 1991b). Tribulin-like activity is not peculiar to man and rat, as it has also been detected in urine from cattle, goats, sheep and pig (Sharman *et al.*, 1987).

Using nonpharmacological, proconvulsant methods Bhattacharya *et al.* (1991a) demonstrated that electroconvulsive therapy (ECT) increased MAO-I in rat brain. They concluded that the antidepressant effect of ECT may be mediated through endogenous inhibition of MAO. Following a similar line of thought, Medvedev *et al.* (1992) studied experimental audiogenic seizures in rats. Weak seizures induced no change in brain MAO-AI or MAO-BI. Moderate seizures induced an increase in both MAO-AI and MAO-BI. Complete tonic epileptiform seizures induced further augmentation of MAO-AI with no further increase in MAO-BI, suggesting distinct inhibiting compounds for MAO-A and MAO-B.

Several of these animal studies described above have demonstrated simultaneous increases in MAO-I alongside reductions in MAO activity, increased monoamines, and/or decreased monoamine metabolites (Armando *et al.*, 1984, 1988, 1989; Clow *et al.*, 1988a; Lemoine *et al.*, 1990, 1994; Hamaue *et al.*, 1992; Medvedev *et al.*, 1994), suggesting *in vivo* MAO inhibition. This was confirmed in a study where the irreversible MAO-I phenelzine was administered to rats with or without concurrent cold-restraint stress. It was found that phenelzine caused significantly less inhibition when given during a period of stress than during a control period (Clow *et al.*, 1989b). Thus, stress can reduce the *in vivo* potency of MAO-inhibitory drugs, a finding compatible with increased production of a competitive endogenous MAO-I.

MAO-I is unevenly distributed in rat tissues (Armando *et al.*, 1986). This work was recently repeated in our laboratory (unpublished observations) using an improved protocol. MAO-AI and MAO-BI were again shown to be unevenly distributed throughout the rat body and the distribution for each activity was dissimilar, suggesting that the two activities do not derive from the exact same substance. Furthermore, both MAO-AI and MAO-BI have now been detected in the adrenal gland (reported absent in Armando *et al.*, 1986). The differential distribution of tribulin activity in rat tissues suggests that it does not stem from some general tissue compound.

Studies in Normal Healthy Humans

It was never clear from this early work, however, whether generation of endogenous MAO inhibitory activity was a pathological response or a normal homeostatic regulatory mechanism. Our group has recently attempted to resolve this problem. We have shown urinary MAO-AI and MAO-BI to correlate with the elevation in arousal experienced by healthy students about to undergo an important assessed oral seminar presentation. Furthermore, MAO-AI correlated with cortisol measured in the same urine samples (Doyle *et al.*, 1996b). In another study, normal individuals were again investigated, this time over a five-day period with no stress challenge. Mean MAO-AI and MAO-BI (across days) were found to be positively correlated with self-reported stress scores over the same time period (Doyle *et al.*, 1996a). This was the first study to demonstrate a relationship between tribulin and "everyday stress." We concluded that tribulin output was indicative of mild "enduring state stress" in normal people and that these levels could be raised even further by an acute stressful episode (as reported in the early literature). As such tribulin could be an endogenous modulator of brain monoamine levels both during resting (everyday) stress and during acute, severe stress. Raised MAO-I activity in reducing monoamine metabolism would increase availability of the monoamines.

To facilitate more detailed examination of this hypothesis, we have developed a sensitive technique to determine MAO-I in human saliva. This technique allows frequent and convenient sampling and overcomes the inherent problems associated with vastly fluctuating diuresis. Using this technique we have again demonstrated an association between MAO-AI and MAO-BI and self-reported stress in normal people (Doyle *et al.*, 1996c).

These studies in normal healthy people have concentrated on the relationship between MAO-I and self-reported feelings of stress (although useful, self-reported measures are inherently subjective). In the light of the known sympathomimetic activity of antidepressant MAO-A inhibitory drugs, we investigated, for the first time, endogenous MAO inhibitory activity and cardiovascular (CV) reactivity. It is known that playing computer games can produce substantial cardiodynamic changes in normal individuals (Miller and Ditto, 1989; Turner and Carroll, 1985), so we choose this as a suitable method of inducing mild, acute psychological stress to investigate these relationships.

We discovered a clear positive correlation between salivary MAO-AI and mean arterial blood pressure (MAP) at the peak of the pressor response (soon after beginning to play the computer game) in normal healthy students. Consistent with this finding, subjects who had high salivary MAO-AI immediately prior to the task went on to have significantly higher MAP throughout the experiment (Clow *et al.*, in press). This study is the first to provide evidence of a relationship between generation of the stress-linked endogenous modulator of monoamine activity (tribulin) and a physiological marker of stress (MAP). It is possible, therefore, that tribulin can increase availability of sympathomimetic agents and is related to CV activity in a similar way to MAO-A inhibitory antidepressant drugs.

Identity of Tribulin

Despite considerable effort, the molecular characterization of the MAO inhibitory component of tribulin has not yet been achieved. 2'3-dioxoindole (isatin) present in urine, brain, cerebrospinal fluid, and other tissues was first thought to account for the MAO-BI of tribulin; $IC_{50} = 3 \mu M$ (Glover *et al.*, 1988). However, recent studies have shown that all the MAO-BI in urine cannot be attributed to isatin alone (Pang *et al.*, 1996). Medvedev *et al.* (1995a) have published evidence that MAO-AI in urine may be attributable to ethyl indole-3-acetate, methyl indole-3-acetate, and 4-hydroxyphenylacetate. However, this remains to be confirmed, as the potency of these agents is low (MAO-A IC_{50} values: $44 \mu M$, $88 \mu M$ and $120 \mu M$ respectively) and they were not detected in brain tissue. Later the same group identified 4-hydroxy phenylethanol as an MAO-A inhibitor in brain; however, the IC_{50} for was even higher at $1.4 mM$ (Medvedev *et al.*, 1995b). To date none of these putative endogenous MAO inhibitors have been shown to be modulated by stress.

Recent studies in our laboratory have shown that the MAO-AI and MAO-BI can be separated as distinct molecular components with aqueous methanol elution from XAD-4 and that the two inhibitory activities have different patterns of distribution in rat tissues (Doyle *et al.*, in preparation).

CONCLUSION

It is known that the monoamines play a key role in both the SAM and HPA stress systems and that small changes in MAO activity have significant effects on

monoamine availability. The body of evidence, taken from a wide range of studies, strongly implies the existence of a regulatory mechanism for MAO, the main metabolic enzyme of these monoamines. The evidence shows that this endogenous inhibitor(s) of MAO (tribulin) is related to "everyday" stress in normal individuals and pathological stress in clinical populations. As such tribulin may play a physiological regulatory role both within the brain, affecting motivational and emotional states, and in the periphery, affecting cardiovascular reactivity. The importance of this regulatory mechanism should not be underestimated.

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3 Glucocorticoids, Serotonin and Their Interactions in the Hippocampus

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GLUCOCORTICOID AND THE BRAIN

The adrenal cortex synthesizes and secretes a range of steroid hormones, most importantly the corticosteroids. Corticosteroids are subdivided into glucocorticoids and mineralocorticoids. Cortisol is the physiological glucocorticoid in humans and corticosterone is its equivalent in rats, whereas there is a single major mammalian mineralocorticoid, aldosterone. Various synthetic glucocorticoids have also been developed, including dexamethasone and prednisolone. Glucocorticoid excess in humans is frequently associated with adverse neuropsychiatric and cognitive effects. This is perhaps best recognized in patients with Cushing's disease, the majority of whom show at least some manifestations of depression and memory dysfunction (Starkman and Scheingart, 1981) but is also seen in patients taking exogenous glucocorticoids, for example as therapy for chronic inflammatory disorders (Von Zerssen, 1976). Glucocorticoids are likely to exert such effects directly on the brain, as these lipophilic steroids readily cross the blood-brain barrier and cell membranes. Thus, in recent years much effort has focused on defining the sites and mechanisms of these actions of corticosteroids upon the CNS.

BRAIN CORTICOSTEROID RECEPTORS

Almost 3 decades ago, Bruce McEwen and his colleagues demonstrated that when [^3H]corticosterone was injected peripherally in the rat there was remarkably high uptake of tracer into the hippocampus (McEwen *et al.*, 1968). This contrasted with much lower uptake in most other brain regions, including the hypothalamus. The poorer retention of [^3H]dexamethasone suggested the hippocampal "corticosterone receptors" differed from classical glucocorticoid receptors in the pituitary and liver, which were known to bind synthetic glucocorticoids like dexamethasone with much higher affinity than corticosterone (De Kloet *et al.*, 1975). Indeed, the

distribution of [^3H]dexamethasone binding in the hippocampus differed from that of [^3H]corticosterone, suggesting that there were at least two distinct types of receptors for glucocorticoids in the brain. A further complexity was the binding of [^3H]aldosterone, which appeared closely to parallel that of [^3H]corticosterone, but showed some unique uptake in specific periventricular areas of the hypothalamus, thalamus and brain stem (McEwen *et al.*, 1986a).

Receptor Structure and Function

A plethora of subsequent biochemical and *in vivo* studies and, more recently, the isolation and cloning of encoding cDNAs, has clearly defined only two subtypes of corticosteroid receptor. These are the type I or mineralocorticoid receptor (MR) and the type II or glucocorticoid receptor (GR) (Evans and Arriza, 1989). Both are members of the superfamily of nuclear steroid–thyroid hormone–vitamin receptors. These receptors are ligand-activated transcription factors, which also include the receptors for estrogen, androgen, progesterone, thyroid hormones, vitamin D, and retinoic acids. The receptors share a common domain structure, with highly homologous regions that bind to DNA and steroids (Evans and Arriza, 1989; Seckl, 1996). GR or MR, when activated by their respective ligands, attach as homodimers (or possibly as heterodimers; see Trapp and Holsboer, 1996) to specific palindromic DNA sequences (glucocorticoid response elements) in the regulatory regions of target genes, affecting transcription (Evans and Arriza, 1989). An alternative mechanism of action that has become apparent in recent years is the interaction of steroid receptors with other transcription factors (such as AP1 or CREB) without direct DNA binding (Pfahl, 1993). The results of such transcriptional control in the brain are alterations in gene expression that impact upon many facets of cellular metabolism, receptor and ion channel density, neurotransmission, and glial function, thus affecting mood, cognition, behavior, neuroendocrine control, as well as cell birth and survival.

Distribution

A series of studies of the two receptor subtypes, using molecular (mainly *in situ* hybridization) and immunohistochemical approaches, has defined their distinctive distributions. GR are very widely expressed throughout the brain, not only in neurons, but also in glia and vascular tissues (McEwen *et al.*, 1986b; de Kloet, 1991; Seckl, 1996). GR are particularly abundant in the hippocampus, cerebral cortex, cerebellum, amygdala, some diencephalic nuclei (e.g., the hypothalamic paraventricular nucleus), and in ascending monoaminergic neurons of the brain stem (e.g., raphe nuclei, locus ceruleus, and nucleus tractus solitarius). Indeed, it is doubtful if any brain cell is entirely devoid of GR. By contrast, MR are at very low density in most brain regions, with high expression restricted to hippocampal neurons (both granular and pyramidal), the septum, and a few brainstem motor nuclei.

Aldosterone-Selective MR: the Role of 11 β -Hydroxysteroid Dehydrogenases

Although two receptors binding similar ligands might seem sufficiently convoluted to accomplish the multitude of corticosteroid actions, the ligand-binding studies outlined above suggest even greater complexity. Thus, there are differences in patterns of binding of dexamethasone, corticosterone, and aldosterone in the brain. *In vitro*, MR bind corticosterone, cortisol, and aldosterone with similar and high affinity, whereas GR bind corticosterone with 10-fold lower affinity, but dexamethasone with very high affinity. *In vivo*, most brain MR reflect the *in vitro* affinities and are occupied by corticosterone (or cortisol), which circulates in 100- to 1000-fold molar excess to aldosterone. The result is that MR are largely occupied by basal corticosterone levels, whilst GR only become substantially occupied during stress or the diurnal maximum. However, some periventricular aldosterone-selective MR also occur (McEwen *et al.*, 1986a). In the distal nephron, selective access of aldosterone to MR *in vivo* is ensured by 11 β -hydroxysteroid dehydrogenase type 2 (11 β -HSD2) which catalyses the rapid metabolism of corticosterone and cortisol to their inert 11-keto products (11-dehydrocorticosterone, cortisone). The brain might also exploit 11 β -HSD2 to produce aldosterone-selective effects not mimicked by corticosterone, such as those on salt appetite and blood pressure (McEwen *et al.*, 1986a). Indeed, inhibition of brain 11 β -HSD, presumably by allowing corticosterone to activate otherwise protected MR, alters functional activity in preoptic and periventricular regions (Seckl, 1997), where normally aldosterone selectively binds and exerts hypertensive effects (Gomez-Sanchez and Gomez-Sanchez, 1992). Although there is widespread 11 β -HSD activity in the brain (Seckl, 1997), this is predominantly the lower affinity type 1 isoform (11 β -HSD1), which appears to regenerate inert 11-keto compounds into the active 11-hydroxy forms in neurons and other tissues (Rajan *et al.*, 1996). However, 11 β -HSD-2 mRNA is expressed in a few periventricular regions in the adult brain (Roland *et al.*, 1995). This presumably underpins the aldosterone-selective uptake and physiological effects, described above.

THE HIPPOCAMPUS

The hippocampus expresses GR and MR at very high density: both receptors are found throughout the hippocampal neuronal layers, with MR evenly expressed and GR mRNA higher in dentate gyrus granule cells and pyramidal cells of CA1 than in CA3. The effects of corticosteroids have also been extensively studied in the hippocampus where they alter electrophysiological activity, biochemical processes, and neurotransmission, thereby affecting mood and behavior, cognitive function, neuroendocrine control and cell survival (McEwen *et al.*, 1986a, 1992; de Kloet, 1991; Jacobson and Sapolsky, 1991; Joëls and de Kloet, 1992; Sapolsky, 1992; Seckl, 1996).

REGULATION OF GR AND MR IN THE HIPPOCAMPUS

Glucocorticoids regulate hippocampal GR mRNA expression and hence receptor protein/binding sites in the short term, although perhaps less so in the longer

term (Seckl, 1996). MR are much less affected by their ligand. Stress also affects hippocampal MR and GR; again the effects are stressor-specific and not usually sustained. These effects are limited to certain hippocampal subregions, underlying the cell-specific nature of GR and MR control. The molecular mechanisms underlying such complex cell-specific regulation are imperfectly understood (Yau and Seckl, 1995). They may reflect varying transcriptional control via multiple tissue-specific promoters of the GR and MR genes and/or posttranscriptional (mRNA stability) and posttranslational (protein phosphorylation, etc.) effects; all have been documented with GR, although MR have been much less studied.

In contrast, neurotransmitters appear to exert potent long-term effects upon hippocampal MR and GR (Yau and Seckl, 1995). Serotonergic (5-HT) neurotransmission is important to maintain normal hippocampal MR and GR density; lesions of 5-HT systems (such as those caused by 3,4-methylenedioxymethamphetamine — “ecstasy” — a common drug of abuse) markedly reduced receptor expression. Loss of the noradrenaline input to the hippocampus similarly reduces MR, but not GR expression. In contrast, cholinergic denervation of the hippocampus increases expression of MR and GR. Dysfunction of both 5-HT and cholinergic inputs to the hippocampus are prominent features of age-related cognitive disorders, including Alzheimer's disease. Combined serotonergic and cholinergic lesions increase GR gene expression selectively in the CA1 subfield, the area most prone to neuron loss in both aged rats and in Alzheimer's disease. Indeed, neuron loss in CA1 correlates with spatial (hippocampus-specific) memory in aged rats.

Molecular Mechanisms of Hippocampal GR Control

Recent studies have started to address the molecular mechanisms whereby neurotransmitters regulate GR and MR gene expression. Glucocorticoids appear to bind to the GR gene, although the precise locus involved remains obscure (Seckl and Olsson, 1995). For serotonin, ketanserin-sensitive 5-HT receptors and cyclic AMP appear to be important (Mitchell *et al.*, 1992), although the precise receptor subtype is unclear, as ketanserin classically is thought to bind to the 5-HT₂ subgroup of receptors that are coupled to phosphatidylinositol rather than cyclic AMP. Perhaps the newly identified 5-HT₆ and 5-HT₇ sites, which couple to cyclic AMP and bind antidepressants including ketanserin, may be important here. Whatever the receptors involved, the subsequent involvement of specific transcription factors with potential DNA binding sites in the GR gene promoter region has also been suggested (Seckl and Olsson, 1995). In particular, NGFI-A (zif268, krox24, egr1) and AP-2 are induced in parallel to increased GR gene expression in specific hippocampal subregions, effects that are in large part prevented by antagonists of 5-HT₂-type receptors (Olsson *et al.*, 1996). This implies a pathway from 5-HT or its release by acute stress and 5-HT₂-like receptors, via cAMP and intracellular calcium release to the synthesis or phosphorylation of specific DNA binding proteins which induce transcription of the GR gene. Similar pathways may operate for MR. Importantly, antidepressant drugs, which amongst other effects, potentiate monoaminergic neurotransmission, appear to have similar actions on GR and MR both *in vivo* and on GR

in neurons *in vitro* (Seckl and Olsson, 1995; Barden, 1996). The role, if any, for nerve growth factor (NGF), which regulates expression of these factors in some cells, remains unclear.

SOME BEHAVIORAL AND NEUROENDOCRINE FUNCTIONS OF HIPPOCAMPAL CORTICOSTEROID RECEPTORS

Hypothalamic–Pituitary–Adrenal (HPA) Axis Regulation

Several studies have implicated the hippocampus as a major suprahypothalamic site of glucocorticoid feedback upon the hypothalamic–pituitary–adrenal (HPA) axis (Jacobson and Sapolsky, 1991). Severing the proposed (indirect) afferent connections to the paraventricular nucleus from the hippocampus increases HPA axis activity in some, but not all, studies (Yau and Seckl, 1995). More importantly, manipulations that decrease hippocampal corticosteroid receptor density without neuronal damage reduce sensitivity to glucocorticoid negative feedback (Jacobson and Sapolsky, 1991). Initial studies using centrally-administered MR and GR antagonists suggested that MR control basal HPA axis activity, whilst GR mediate termination of the stress response (Ratka *et al.*, 1989). However, the requirement for additional corticosterone to reduce HPA activity at the diurnal peak indicates a shift in control from MR to GR, and recent findings again highlight interactions between receptors (Bradbury *et al.*, 1994), with MR occupancy apparently necessary for GR-mediated effects to be fully manifest. The molecular basis for this observation remains to be determined (perhaps MR:GR heterodimerization may play a role).

However the proposed role of glucocorticoids in the hippocampus to inhibit the HPA axis has an apparent paradox. Thus, elevated glucocorticoid levels acting via GR, in general, act to inhibit electrical activity in the hippocampus (Oitzl and de Kloet, 1992). If lesioning the 'pathway' from the hippocampus to paraventricular nucleus, which is the common efferent output of the brain to the HPA axis, leads to HPA activation, then how can glucocorticoid suppression of hippocampal electrical activity have similar stimulatory effects? While this point remains to be fully resolved, the following issues illustrate the complexity of the system that may account for the apparent "paradox." First, the hippocampus is not a homogenous structure, but is comprised of complex interprojecting sheets of neurons. These do not subserve a single monolithic function, as recently illustrated for cognitive behaviors. Stimulation of the various subfields may stimulate or inhibit the HPA axis (Dunn and Orr, 1984). Thus, the electrophysiological studies in one subfield may not reflect the net effect and output over the whole hippocampal structure. Second, the pathways from the hippocampus to the hypothalamic paraventricular nucleus are largely indirect and involve inhibitory effects upon relays in the bed nucleus of the stria terminalis and the amygdala (Herman *et al.*, 1994). Thus, a "double-negative" effect may be pertinent: glucocorticoid inhibition of the hippocampus attenuating an inhibitory influence that synapses with an inhibitory GABA neuronal pathway to the paraventricular nucleus of the hypothalamus. The effects of lesions under such circumstances will be determined by the site and extent of effects upon the other

paths and nuclei involved. Third, it seems unreasonable to expect that a single limbic site will represent *all* the suprahypothalamic negative control of the efferent glucocorticoid stress response; other regions express GR and MR and their influences will not be altered by hippocampal manipulations. Thus, the hippocampus is likely to be an important locus for glucocorticoid control of the hypothalamic–pituitary–adrenal stress response.

Glucocorticoids and Memory

Glucocorticoids modulate cognitive function in rats (Oitzl and de Kloet, 1992) and in humans (Newcomer *et al.*, 1994). In the absence of hippocampal damage, the mechanism may reflect glucocorticoid-mediated effects on hippocampal neuronal excitability (Joëls and de Kloet, 1992b) and/or long term potentiation (LTP; an electrophysiological correlate of memory), which itself correlates directly with learning. Recent studies show an “inverted U-shaped” relationship between glucocorticoid levels and LTP in rats (Diamond *et al.*, 1992; Kerr *et al.*, 1994). Low or basal levels of glucocorticoids or MR agonists potentiate LTP. By contrast, high glucocorticoid levels, stress or GR agonists reduce LTP and attenuate memory. Consistent with these electrophysiological data, there is a highly significant negative correlation between corticosterone levels and spatial memory in aged rats (Yau *et al.*, 1995).

INTERACTIONS BETWEEN 5-HT AND GLUCOCORTICIDS IN THE HIPPOCAMPUS

A popular notion is that disordered 5-HT neurotransmission causes affective disorders (Meltzer and Lowy, 1987). However, Cushing’s disease patients are usually depressed and, intriguingly, patients with primary major affective disorders often show increased glucocorticoid (cortisol) levels and insensitivity to feedback suppression of the hypothalamic–pituitary–adrenal axis. Indeed, elevated cortisol levels precede depressive episodes in recurrent depression (Seifritz *et al.*, 1995), suggesting a possible causal role. This notion has been supported by several recent, albeit limited, studies that have reported the successful use of inhibitors of adrenal synthesis of cortisol, such as metyrapone and ketoconazole, in patients with depression resistant to more conventional agents (Dinan, 1994). Thus, there has been intense interest in interactions between the 5-HT and glucocorticoids in the brain. The hippocampus is frequently employed as an archetypal region for such interactions, although clearly several other brain regions, such as the prefrontal cortex, are also of importance for various behaviors and pathologies, including depressive illnesses.

5-HT Receptors and Glucocorticoids

There is a dense 5-HT innervation of the hippocampus from the midbrain raphe nuclei. As outlined above, this exerts an important influence upon hippocampal GR

and MR density. Glucocorticoids also influence 5-HT biosynthesis in the raphe and 5-HT turnover, receptors, metabolism and reuptake in the hippocampus and other target structures (McEwen, 1987; de Kloet, 1991). In particular, corticosterone, acting via GR, appears to be necessary, at least in part, for the stress-induced release of 5-HT (Chaouloff, 1993; Singh *et al.*, 1994). Multiple 5-HT receptor subtypes are expressed in the hippocampus. Several investigators have examined the effects of adrenal steroids upon 5-HT_{1A} receptors in the hippocampus. This receptor subtype is of particular interest as it has a proposed role in depressive and anxiety disorders. Acutely, glucocorticoids acting via GR stimulate postsynaptic electrophysiological responses to 5-HT_{1A} receptor activation, whereas lower doses of corticosterone acting via MR have the opposite effect, inhibiting 5-HT_{1A} receptor effects (Joëls and de Kloet, 1992a). In congruence with the latter findings, many studies have shown that 5HT 1A receptor-gene expression and binding sites in the hippocampus are induced by adrenalectomy and attenuated by low doses of corticosterone (acting via MR), although the precise locus and magnitude of these effects vary considerably between studies (Chalmers *et al.*, 1993; Meijer and de Kloet, 1993); one group found no change with corticosteroid manipulations, at least at the level of 5-HT_{1A} receptor mRNA (Holmes *et al.*, 1995b). The discrepancies may reflect the different rat strains used (genetics) or the conditions in which the animals were maintained (environment). Either or both processes are known to influence basal corticosterone levels, which if persistently elevated may have downregulated the potentially dynamic 5-HT_{1A} receptor response. However, the true basis for this variation remains undetermined and might, in part, reflect some of the crucial processes underlying the variation in resistance or sensitivity of particular individuals to affective dysfunction under particular environmental circumstances.

Glucocorticoid control of the other subtypes of 5-HT receptors that are highly expressed in the hippocampus has been less intensively studied. However, the 5-HT_{2C} receptor subtype, which appears to exert rather negative effects upon mood and behavior whilst stimulating the hypothalamic-pituitary-adrenal axis, is also potently suppressed by glucocorticoids, this time acting largely via GR (Holmes *et al.*, 1995a,b). Antagonists of the 5-HT_{2C} receptor are atypical antidepressants and here glucocorticoids normally appear to suppress transmission. Whether failure of this apparently protective control may occur in affective disorders, perhaps leading to a mismatch between 5-HT_{2C} receptor activation and GR effects, remains an intriguing area for investigation. Other 5-HT receptors are also affected by corticosteroids, including the 5-HT₆ and 5-HT₇ subtypes (Yau *et al.*, 1996), but the importance of these binding sites, although intriguing since they bind antidepressant drugs with high affinity, remains obscure.

Antidepressant Drugs

In vivo, antidepressant drugs, which amongst other actions potentiate monoaminergic (e.g., 5-HT and noradrenaline) neurotransmission, increase GR and MR in the hippocampus, thus enhancing glucocorticoid feedback sensitivity (Seckl and Olsson, 1995; Barden, 1996; Holsboer and Barden, 1996). Similar effects are seen with antidepressant administration to transgenic mice with reduced central GR expression,

which may model the neuroendocrine features of depression and aging (Pepin *et al.*, 1993). These effects are also seen *in vitro* and may in part reflect direct effects upon the target hippocampal neurons. 5-HT also increases GR expression directly in hippocampal neurons in culture, emphasizing the potency of this control of receptor expression (Mitchell *et al.*, 1990). Antidepressants also reverse the impairment of spatial memory following stress and the adverse effects of glucocorticoids upon hippocampal dendritic structure (McEwen *et al.*, 1993; Luine *et al.*, 1994). Given the links between glucocorticoid hypersecretion, cognitive impairment, and hippocampal pathology, treatment with antidepressants that correct glucocorticoid hypersecretion may be of therapeutic value. Intriguingly, there is a direct correlation between antidepressant-mediated increases in hippocampal MR mRNA expression and improved cognitive performance in young rats (Yau *et al.*, 1995). Aged rats lose these plastic responses of hippocampal MR and memory to antidepressants (Yau *et al.*, 1995). Whether earlier treatment may prevent or ameliorate age-related cognitive decline and HPA axis dysfunction remains to be explored. In contrast, the enhancement of glucocorticoid feedback sensitivity with antidepressant treatment is maintained in old rats, perhaps because loci other than the hippocampus are involved in this effect (Holsboer and Barden, 1996).

GLUCOCORTICIDS AND THE HUMAN BRAIN

Glucocorticoid feedback insensitivity in chronically-stressed nonhuman primates has been associated with a selective decrease of hippocampal GR (Brooke *et al.*, 1994). Stress predisposes to human depression, and this model may explain the hypercortisolism and glucocorticoid resistance in human affective disorders. GR and MR mRNAs are also highly expressed in the human hippocampus (Seckl *et al.*, 1993), suggesting this may also be a major target for glucocorticoid action. As in rats, elevated glucocorticoids attenuate hippocampal memory in humans (Newcomer *et al.*, 1994). Importantly, hypercortisolemia correlates with age-related cognitive decline (Lupien *et al.*, 1994). Patients with elevated cortisol levels due to Cushing's syndrome also exhibit impairments in cognitive function. Hippocampal volume correlates positively with memory and negatively with cortisol levels (Starkman *et al.*, 1992). Extrapolating from animal studies, it is plausible that in Cushing's disease or in 'unsuccessful' cognitively-impaired human aging, elevated glucocorticoids act via hippocampal GR to attenuate electrophysiological function and hence memory.

The hippocampus is also consistently an early and prominent target for damage in Alzheimer's disease. Loss of hippocampal volume correlates with glucocorticoid feedback insensitivity (De Leon *et al.*, 1988). Hypercortisolism and glucocorticoid feedback insensitivity are frequent in Alzheimer's disease and rising cortisol levels in early Alzheimer's disease predict memory deterioration (Craft *et al.*, 1993). Thus, aged humans and rats show qualitatively similar adrenocortical hyperfunction. Recently, hippocampal MR and GR gene expression was found to be unaltered in surviving neurons in Alzheimer's disease, despite marked glucocorticoid hypersecretion (Seckl *et al.*, 1993). Presumably, the primary Alzheimer's disease process causes bulk loss of hippocampal neurons, producing insensitivity to glucocorticoid feedback

and hence hypercortisolaemia. The maintenance of high GR expression in surviving cells, perhaps in part a consequence of loss of the normal regulatory innervations, may then allow full transduction of the deleterious effects of the glucocorticoid excess. It remains to be determined whether reduction of glucocorticoid levels (e.g., with metyrapone) might improve memory dysfunction in Alzheimer's disease (which seems unlikely since neuron loss has occurred) or in otherwise normal elderly subjects with elevated glucocorticoid levels and poorer cognitive performance (Lupien *et al.*, 1994) (which is perhaps more probable, as this may represent reversible dendritic contraction and neuronal dysfunction rather than death). Such approaches have been anecdotally reported as beneficial in severe depression (Murphy, 1991) in which adrenal enlargement, hypercortisolaemia and feedback insensitivity are also prevalent, particularly in the elderly (Maes *et al.*, 1990). Use of antidepressants in human cognitive dysfunction with age has also been mooted, with some possible improvements reported in some series (Oxman, 1994). However, much requires to be clarified before the animal data can be extrapolated with any confidence to humans.

CONCLUSION

Glucocorticoids, acting via both MR and GR, exert myriad effects in the brain. Decreased hippocampal GR expression may, in part, underlie HPA hyperactivity frequently associated with neuropsychiatric disorders such as depression and cognitive decline. Pharmacological modulation of brain corticosteroid receptors (e.g., using antidepressants) increases hippocampal receptor expression and improves cognitive and HPA function in the rat. Further studies in nonhuman primates and humans are required to determine whether these approaches may be of importance to disorders of hippocampal function in humans.

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4 Brain Corticosteroid Receptors: Behavioral and Neuroendocrine Aspects

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Corticosteroid hormones modulate behavior, influence cognitive functions, and exert neuroendocrine control through binding to two intracellular receptors in the brain: the high affinity mineralocorticoid receptors (MRs) and the low affinity glucocorticoid receptors (GRs; De Kloet *et al.*, 1993, 1995; McEwen and Sapolsky, 1995). Corticosterone is the main glucocorticoid in rats and mice. In contrast to MRs, GRs are widely distributed throughout the brain and their site of action might differentially influence the neuroendocrine and behavioral responses. Moreover, the action of corticosteroids is conditional, has certain temporal features, and is context dependent. The hippocampus can be regarded as the main brain area involved in the behavioral effects of corticosteroids. Neuroendocrine regulation is primarily attributed to the hypothalamus and pituitary, but also to the hippocampus (Herman *et al.*, 1989). This contribution focuses on the role of MRs and GRs, the plasma levels of ACTH and corticosterone and information processing at the behavioral level. First, I will present some neuroendocrine and behavioral examples on MR- and GR-related functions. Of special interest is the predictive value of a limited amount of neuroendocrine data for behavior. Is it possible to relate the behavioral output to the level of corticosteroids (and ACTH) measured in the blood? Does the latter specifically reflect the activation and functionality of brain MR and/or GR? How might acute and chronic changes of the corticosteroid system like chronically elevated levels of corticosteroids, alterations in the functionality of the receptors influence behavior? These and other points will be addressed in the final part.

NEUROENDOCRINE REGULATION

Activity of the HPA Axis: MR- and/or GR-mediated as well as Brain-site Related Effects

Pharmacological blockade of MRs and GRs (see Table 1; Ratka *et al.*, 1989; van Haarst *et al.*, 1996, 1997) as well as adrenalectomy (ADX; Dallman *et al.*, 1994) were

Table 4.1 Treatment with a MR antagonist (MRa; RU28318) and a GR antagonist (GRa; RU38486) at the nadir (a.m.) and peak (p.m.) of the circadian rhythm of the HPA axis in young male Wistar rats. Drugs were injected into the lateral ventricle (icv) or into the dorsal hippocampus (hpc) or chronically infused icv. Blood samples were taken 60 min after treatment. Animals were treated and placed into a novel environment (challenge) and blood was collected sequentially at different timepoints. Data from Ratka *et al.* (1989), van Haarst *et al.* (1996, 1997), compared to vehicle control group: ↑ increase, ↓ decrease, = no change

	<i>a.m./basal</i>		<i>a.m./challenge</i>		<i>p.m./basal</i>	
	<i>ACTH</i>	<i>CORT</i>	<i>ACTH</i>	<i>CORT</i>	<i>ACTH</i>	<i>CORT</i>
<i>Acute icv</i>						
MRa	↑	↑	↑	↑	↑	↑
GRa	=	=	↑	↑	↑	↑
<i>Chronic icv</i>						
GRa						
day 1	=	=			↑	↑
day 2	=	=	↑	↑	=	↑
> day 3	=	=			=	↑
<i>Acute hpc</i>						
MRa					↑	
GRa					=	↓

used to demonstrate the specific MR and GR-mediated effects on the activity of the hypothalamic-pituitary-adrenal (HPA) axis. ADX produced a marked and sustained increase in ACTH secretion, upregulation of both neuropeptides, CRH and AVP. When ADX rats were treated with corticosterone in amounts that produce plasma corticosterone concentrations that can primarily occupy MRs in the brain, the ADX-induced disinhibition of the HPA axis was restored during the trough of the circadian rhythm to the level observed in adrenally intact rats. Additional GR activation was required for restraining the circadian peak and stressor-induced ACTH secretion. Tonic elevations of corticosterone in the range in which both MRs and GRs were occupied completely inhibited the endogenous activity of the HPA system. Specific antagonism of brain MRs and GRs revealed that both receptors are involved in the stress-induced corticosterone response. At the trough of the circadian rhythm, MRs alone restrained the release of corticosterone whereas at the peak GRs are involved as well. Thus, two different approaches revealed the same picture of MR and GR mediated control of the HPA axis: under basal resting conditions MR activation is sufficient to suppress HPA activity, whereas an extra stimulation of the system requires the balanced and coordinated action of MRs plus GRs.

To mimic GR resistance a competitive GR antagonist (RU38486) was infused over days into the brain (van Haarst *et al.*, 1996). This resulted in the expected stress-induced prolonged activation of the HPA axis (elevated levels of ACTH and corticosterone). The diurnal rhythm of ACTH and corticosterone was affected differently. The basal tonus of the system in the morning did not change. In the evening of the first day of infusion corticosterone and ACTH were increased, but surprisingly, on

the following evenings corticosterone levels only were found to be elevated. An *in vitro* assay revealed that the adrenals had become more sensitive to ACTH.

Most recent findings on a brain-site related differential effect of GR activation add to the complexity of the system (van Haarst *et al.*, 1997). In contrast to the known hypothalamic and pituitary GR-mediated negative feedback action, hippocampal GRs appeared to activate the HPA axis. Injection of the GR antagonist into the dorsal hippocampus resulted in a suppression of ACTH, whereas intracerebroventricular (icv) injection of the drug elicited an elevation of ACTH. Since both hippocampal and icv injection of an MR antagonist (RU28318) resulted in an increase of ACTH and corticosterone, the suppressive action of MRs on the HPA is considered a predominant hippocampal effect. We have shown that the higher capacity of hippocampal MRs in Lewis rats is related to the restraining action of MRs on the HPA axis (Oitzl *et al.*, 1995). Thus, in the hippocampus GRs exert effects opposing the MR-mediated actions of this brain area. Furthermore, a potentiated activation of hippocampal MRs might be easily misjudged as a disturbed GR feedback at the hypothalamic-pituitary level.

This underlines the pivotal role of a balanced MR/GR activation and put forward the additional relevance of brain-site specific activation and concerted action of GRs for neuroendocrine control.

BEHAVIORAL REGULATION

Behavioral responses themselves are thought to be represented centrally in a set of hierarchically organized feedback systems. The capacity to organize and synchronize the various systems is largely ascribed to the hippocampus (Gray, 1995; Eichenbaum and Otto, 1994). One effect of corticosteroids is to alter the response characteristics of hippocampal neurons (Joëls and de Kloet, 1994; see Joëls, this volume). It is reasonable to assume that the relative MR/GR activation in the hippocampus will, with respect to time of action, context, and duration, modulate the processing of events within the hippocampal networks. The consequences of these information processing will finally be reflected in behavior.

A challenge, i.e., an external or internal event and thus also training trials in learning tasks, triggers HPA activity and corticosteroids are released. Pharmacological and endocrine approaches in combination with a task strongly related to undisturbed hippocampal functioning (i.e., the Morris water maze; Morris *et al.*, 1982) provided a valuable tool to unravel the role of corticosteroids and MR/GR-activation for behavior (Oitzl and de Kloet, 1992). Variations of the time of treatment with respect to underlying processes of learning and memory (e.g., pretraining, posttraining, and preretrieval application of a receptor antagonist) and close observations of the animals' behavior allowed to elucidate the contextual and temporal action of GRs and MRs. GRs were found to be involved in the consolidation of memory (long-term memory formation), whereas MRs were involved in the behavioral pattern. As also demonstrated by inhibitory avoidance tasks, activation of MRs is particularly relevant for the behavior preceding the storage of information (Oitzl *et al.*, 1993; Sandi and Rose, 1994a,b) and thus, can indirectly result in alterations of memory.

In summary, MRs affected the behavior in novel situations. In terms of learning and memory, this is the time of acquisition. Depending on the requirements of a task, different behavioral pattern during acquisition (e.g., alterations in search strategy in rats, pecking behavior in chicks) will consequently influence the storage of the events, a GR-mediated effect.

In line with the behavioral effects of acute GR blockade, we expected that chronic GR antagonism via icv infusion would result in impaired spatial learning. Surprisingly, spatial memory of these rats was improved (Figure 4.1; Oitzl, unpublished results). Furthermore, swim patterns pointed to an increased participation of MRs in the behavior of these animals. The apparent paradox of this finding will be discussed below.

Administration of a certain amount of exogenous corticosterone in context with the performance in a learning task and with certain temporal aspects, i.e., only in close relation to the training trial and in a dose-related fashion, clearly potentiated memory formation (for review see McEwen *et al.*, 1986; Sandi and Rose, 1994a; Flood *et al.*, 1996). Most recently, Sandi and coworkers (1997) showed in an elegant

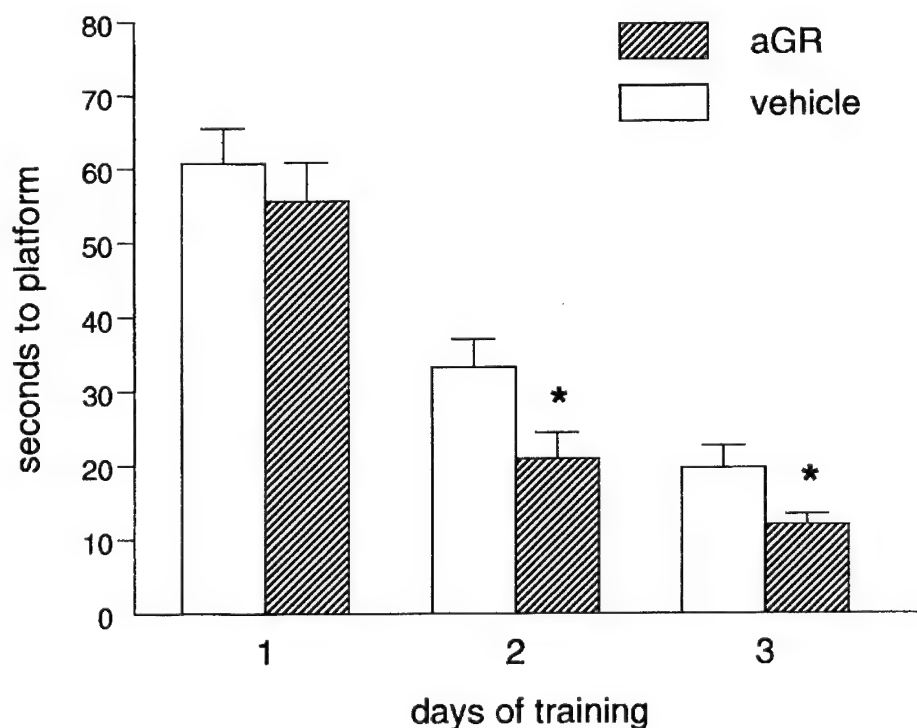


Figure 4.1 During the four trials per day the rats had to locate the submerged platform in a fixed location of the water maze. Rats were similar on the first day, i.e. comparable acquisition. Animals receiving the GR antagonist (RU38486, 100 ng/0.5 l/hour; $n=8$) chronically infused into the lateral ventricle improved their performance on the second and third day of training compared to vehicle infusion controls ($n=12$), i.e. facilitation of consolidation. Data are given as mean seconds per trial + SEM. * $P<0.05$.

study that corticosterone given after the task in a comparable amount as elicited by experience facilitated spatial memory.

Contradictory findings exist on the cognitive effects of chronically increased levels of corticosterone. Most dramatic behavioral deficits were reported in older animals treated with corticosterone or animals subjected to social stress (Sapolsky *et al.*, 1986; Bodnoff *et al.*, 1995) and in young animals in combination with neurotoxic agents (Bennett *et al.*, 1996). Young animals often did not respond to long-lasting (exogenous) elevation of corticosterone; in some cases they even improved their performance (Bodnoff *et al.*, 1995; Luine *et al.*, 1996). These findings extend the importance of the conditional and context-dependent properties of corticosteroid action. However, high corticosteroids presumably represent the risk factor acting synergistically in a disbalanced organism (see also review: McEwen, 1995; McEwen and Sapolsky, 1995). Unfortunately, the in-depth discussion of this topic is not within the scope of this contribution (see Levy, this volume).

Taken together, transient and context-related activation of GRs during and after acquisition has clearcut positive, facilitating effects on the neural mechanisms involved in memory. The same conclusion was drawn from "deficit" models where corticosteroids cannot exert their action due to blockade of their receptors and the absence or reduction of circulating corticosteroids.

INTEGRATION OF NEUROENDOCRINE AND BEHAVIORAL CORTICOSTEROID RECEPTOR-MEDIATED EFFECTS

The balance between MR- and GR-mediated effects is of paramount importance for the homeostatic control of the animal's stress responsiveness, adaptation and cognition (de Kloet *et al.*, 1993). If the MR/GR activation ratio is shifted, the control of corticosteroids on neuronal excitability, neuroendocrine reactivity, and behavior will change. Another important consideration for receptor changes is that the two receptors interact. Moreover, preventing the activation of one receptor such as GR will in addition to the loss of GR-related effects be accompanied by a predominant MR activation and its effects. The dynamics of these processes are largely unknown.

Facilitation of Memory: Adequate and Acute High Corticosteroids and GR Activation

In an intact organism an increased amplitude of corticosterone will transiently activate GR and suppress the activity of the HPA axis. This effect will be potentiated by the additional injection of exogenous corticosterone. It can be assumed that the time window of GR activation in brain structures related to learning and memory will be in the optimal range for the consolidation of preceding events. The result is the facilitation or potentiation of memory formation.

Impairment of Memory: Increased Levels of Corticosteroids Mimicking Partial GR Resistance Due to Acute GR Antagonism

GR antagonism resulted in a prolonged elevation of corticosterone and ACTH, indicating the diminished negative feedback-activity at the hypothalamus and

pituitary. Due to the competitive nature of the antagonist, the drug and the endogenous agonist might variably prevent activation or activate the receptor, respectively. As the effect duration of the antagonist is unknown, prolonged high levels of corticosteroids can have deleterious effects as well. In both situations, temporal and contextual aspects of GR activation are not given and processing of information might be prevented or be strongly disturbed. The result is an impairment of memory processes.

Facilitation of Memory: Increased Levels of Corticosteroids Mimicking GR Resistance Due to Chronic GR Antagonism

The neuroendocrine profile of acute and chronic icv GR antagonist administration in response to the novel environment challenge indicated a loss or disturbance of the negative feedback. In both situations, the peak amplitude of the response was lower and followed by sustained, elevated levels of ACTH and corticosterone compared to the vehicle control groups. Concurrently, the adrenals had become more sensitive in the chronic GR antagonist condition. The blunted peak amplitude suggests a prominent involvement of MRs. More expressed MR-related behavioral effects like the persistent search strategy were observed in later stages of chronic GR antagonism. Thus, neuroendocrine and behavioral data indicate a shift towards a more prominent MR/GR activation. It is suggested that continuous infusion of the GR antagonist might have protected neurons in brain areas involved in learning and memory processes from overexposure to high levels of corticosteroids. At the same time, hypothalamic and pituitary GR-mediated negative feedback was prevented. Alternatively, as the facilitation of memory by corticosteroids is a dose-related effect, the critical amount of corticosterone might have activated GR and thus potentiated memory formation. Unfortunately, it is impossible to control the extent of GR blockade in different brain areas and the adaptive changes of the corticosteroid system in relation to performance *in vivo*. Dose-dependent effects of GR antagonism and direct hippocampal injections of the GR antagonist will be the subject of future studies.

Taken together, prolonged high levels of circulating corticosteroids indicate a disturbance of receptor function which might be MR and/or GR related. Receptors at different brain sites might be differentially affected. If contextual and temporal factors are taken into account, there is no doubt that corticosteroids have positive effects on memory. The balance of receptor-mediated effects depends on the receptor properties and the ligand concentration. Both are influenced by a series of variables. For example, changes in the MR/GR balance can be: genetically determined; induced by early life experiences; altered by age and disease; and can be induced pharmacologically. Concurrent biochemical and behavioral characteristics of the corticosteroid system have been demonstrated. With respect to the data and ideas discussed I will propose a question to the reader: What kind of relationship might exist between a decreased capacity of hippocampal GRs and the concomitantly occurring high circulating levels of corticosteroids and behavioral deficits? This altered capacity might be an adaptive response and thus present protection against long-lasting activation by corticosteroids. However, it might be the negative

consequence of excessive (duration and/or intensity) corticosteroid exposure as well. In fact, when and how a "healthy" corticosterone signal turns to be a "damaging" one is presently unsolved (de Kloet, 1995).

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5 The Role of Brain-Derived Neurotrophic Factor in the Central Effects of Stress

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Stress can have a profound impact on brain and behavior. Stressful events can produce very vivid memories or be suppressed for a lifetime. Humans and other animals can habituate to repeated stressors or can become sensitized to a particular stressor. Stress can produce brain damage. Stress can precipitate psychiatric illness in vulnerable individuals. But how stress alters brain structure and function is not well understood. In this chapter, we consider the possibility that neurotrophic factors might mediate some of the long-term effects of stress on brain function.

EFFECTS OF STRESS ON BRAIN STRUCTURE AND FUNCTION

The hippocampus, which is important in memory and the regulation of the hypothalamic–pituitary–adrenal (HPA) axis, is particularly vulnerable to stress-induced damage. For example, administration of corticosterone to rats at doses that produce blood levels similar to those induced by stress caused a loss of neurons that was especially apparent in the CA3 pyramidal layer of the hippocampus (Sapolsky, 1992). Monkeys also showed evidence of stress-induced neuronal damage in the CA3 and CA4 layers of the hippocampus in response to chronic stress (Uno *et al.*, 1989).

Shorter periods of stress or glucocorticoid administration produce more subtle changes in hippocampal morphology. For instance, 21 days of stress or glucocorticoid administration can cause atrophy of dendrites on CA3 pyramidal neurons in the hippocampus (Wooley *et al.*, 1990; Watanabe *et al.*, 1992). In addition, 21 days of stress or glucocorticoid treatment also produced early signs of neuronal degeneration such as shrunken cells in the CA3 region. Dentate granule neurons and CA1 pyramidal neurons were unaffected by these treatments.

Morphological changes in the hippocampus have functional consequences. For example, stress, like aging, impairs memory tasks dependent on hippocampal function. Chronic stress impaired spatial memory assessed by the Morris water maze

in which rats must find and remember the location of a submerged platform (Bodnoff *et al.*, 1995). Moreover, neuropsychological impairments occur more frequently in patients with posttraumatic stress disorder (PTSD). For example, POWs from the Korean War (86% of whom had PTSD) had significantly more problems with short-term memory assessed by the Wechsler Memory Scale compared to combat veterans, only 14% of whom had PTSD (Sutker *et al.*, 1991). Likewise, Vietnam veterans with combat-related PTSD scored significantly lower on the Wechsler Memory Scale compared to matched controls. Political prisoners who were physically and psychologically tortured had more problems with memory and concentration compared to nontortured prisoners (Basoglu *et al.*, 1994). Interestingly, recent evidence suggests that some PTSD patients with short-term memory deficits have an atrophied hippocampus based on MRI measurement (Bremner *et al.*, 1995).

NEUROTROPHIC FACTORS

Because neurotrophic factors are necessary for the normal development, survival and plasticity of neurons, we hypothesized that stress or corticosterone might decrease the expression of neurotrophic factors that would cause or exacerbate the neuropathological effects of chronic stress in the central nervous system (CNS). Neurotrophic factors are humoral substances that promote the growth and differentiation of neurons (Lindsay *et al.*, 1994). Several new members of the nerve growth factor (NGF) family have recently been cloned, including brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4 (NT-4). BDNF is found throughout the adult brain and may be trophic for a wide variety of neurons. The administration of BDNF dramatically increases the expression of neuropeptides (Nawa *et al.*, 1994) and neurite outgrowth and branching (Patel *et al.*, 1995). Neurotrophic factors are classically thought to be released from a postsynaptic neuron, bind to one of several tyrosine receptor kinases (Trks) on the surface of the presynaptic neuron, and then retrogradely transported to the nucleus where they influence gene expression. In this regard, neurotrophic factors can be thought of as intercellular messengers that allow a target neuron to regulate gene expression in the neurons that innervate it.

Various traumatic insults induce dramatic changes in the expression of neurotrophic factors in the CNS (for review see Lindvall *et al.*, 1994). Seizures increase BDNF mRNA in the hippocampus and cortex. Similarly, ischemia or hypoglycemia induces a transient increase in BDNF mRNA. The direction of these observed changes in BDNF mRNA levels during seizures, ischemia and hypoglycemia is consistent with the notion that glutamate, which is released during brain injury, positively regulates BDNF expression (Zafra *et al.*, 1992).

EFFECTS OF STRESS ON BDNF mRNA EXPRESSION

To explore the relationship between stress and neurotrophic factors, we subjected rats to various stressors and measured changes in neurotrophic factor expression by

in situ hybridization. Because BDNF affects neuronal function and morphology of cultured hippocampal neurons, we were interested whether stress might decrease BDNF mRNA expression in the hippocampus or elsewhere in the rat brain.

We found that immobilization stress for 2 hours decreased BDNF mRNA in the hippocampus of adult rats as measured by *in situ* hybridization (Smith *et al.*, 1995). The most profound decrease occurred in dentate gyrus granule neurons. However, BDNF was also decreased by stress in CA3 and CA1 hippocampal pyramidal neurons, and repeated stress caused decreases in BDNF in other limbic areas such as the basolateral amygdala. High doses of corticosterone also decreased BDNF but only in the dentate gyrus. Interestingly, chronic treatment with antidepressants or electroconvulsive seizures prevent the stress-induced reduction in BDNF (Nibuya *et al.*, 1995).

In contrast to the stress-induced decreases in BDNF expression in the hippocampus and limbic areas, stress *increased* BDNF mRNA levels in the hypothalamus and pituitary (Smith *et al.*, 1995). BDNF was colocalized with corticotropin-releasing factor and thyrotropin-releasing hormone in hypothalamic PVN neurons. Moreover, adrenalectomy or thyroidectomy increased BDNF mRNA levels in the hypothalamus and pituitary. The role of BDNF in the HPA axis is unknown. Possibilities include trophic effects on pituitary cells, regulation of peptide secretion, or even release into the general circulation as a hormone.

Aged animals are more vulnerable to the damaging effects of chronic stress. One possible explanation for an age-related decline in brain function would be a decrease in the availability of neurotrophic factors. Therefore, we addressed the question whether the magnitude of the stress-induced decrease in BDNF expression might be greater in older animals. Contrary to our prediction, however, the ability of stress to modulate BDNF mRNA levels in old Fischer 344/N rats (24 mo) was significantly attenuated both in the hippocampus (a smaller decrease in BDNF) and the PVN (a smaller increase in BDNF) compared to young (3 mo) rats (Smith *et al.*, 1996). Perhaps decreases in BDNF induction may pertain to decreased neural adaptation in the aged brain. Failure to induce growth factors during critical times might also reduce the chance of recovery and prevent a return to homeostasis in aging. Whether reduced expression of BDNF may contribute to age-associated impairments in learning and memory remains to be determined.

Alteration in growth factor levels are particularly relevant to development and would be predicted to have the greatest impact on subsequent brain function and behavior. Prolonged maternal separation interferes with normal growth and development and is a significant risk factor for adult psychopathology. In rats, maternal deprivation can disinhibit the HPA axis which is normally quiescent during the "stress-hyporesponsive period" from day 4–14. In an effort to understand how maternal deprivation may alter HPA axis sensitivity, we used *in situ* hybridization to measure changes in the expression of neurotrophins in rats (postnatal day 12 and 20) after 24 hours of separation from their mother. Preliminary evidence suggests that BDNF was reduced by maternal deprivation in the hippocampus at postnatal day 12. Alterations in neurotrophic factors during critical developmental periods likely influences processes such as apoptosis and neurite branching and thereby alters neural connectivity. Maternal deprivation during the stress hyporesponsive

period in neonates may have long-term consequences for regulation of the HPA axis and may lead to abnormal behavior by permanently increasing stress sensitivity.

CONSEQUENCES OF STRESS-INDUCED CHANGES IN BDNF

These results raise the possibility that some of the effects of stress on brain structure and function could be mediated in part by changes in neurotrophic factor expression. During postnatal development this could influence granule cell number and the extent of their connections with the entorhinal cortex and CA3 pyramidal neurons. In the mature CNS, the consequence of reduced BDNF may be atrophy rather than death of neurons. Destruction of the hippocampus, which abolishes target-derived neurotrophic factors, produces atrophy but not death of adult septal cholinergic neurons for up to 500 days after the lesion (Sofroniew *et al.*, 1993). Thus, a reduction in BDNF may not be sufficient by itself to cause the death of hippocampal neurons during aging or chronic stress, but it could make neurons more vulnerable to injury.

The fact that stress or corticosterone not only produce deleterious effects on CA3 dendrites and cell viability, but also decrease BDNF expression in the hippocampus, suggests that reduced BDNF availability might contribute to the pathological effects produced by stress or high levels of corticosterone. Withdrawal of BDNF could contribute to the reduction in CA3 apical dendrites observed during chronic stress. If a reduction in BDNF causes retraction of axons, then stress might lead to a retraction of mossy fiber axons from the apical dendrites of CA3 hippocampal neurons.

A reduction in BDNF might not only affect the brain structurally, but may also have profound consequences for signal transmission between neurons. For example, in contrast to the inhibitory effects of stress and high dose glucocorticoids on hippocampal excitability, BDNF increases hippocampal excitability and long-term potentiation (LTP), a model of learning and memory. BDNF increases both presynaptic firing and the magnitude of the postsynaptic response in cultured hippocampal neurons (Levine *et al.*, 1995). BDNF induces a relatively long-lasting form of LTP in CA1 neurons distinct from that induced by NMDA (Kang *et al.*, 1995). Moreover, LTP induction is markedly impaired in mice lacking BDNF (Korte *et al.*, 1995). Thus, a reduction in BDNF in the hippocampus during stress may decrease LTP and may interfere with the acquisition and consolidation of memory.

Changes in growth factor expression must now be added to the cascade of changes that occur in response to stress. An interesting quality about growth factors is that they are theoretically capable of translating environmental stressors into structural and functional brain changes, thereby causing long-term changes in behavior. Elucidating the role of growth factors in stress may contribute to our understanding of how a prior history of stress or trauma can alter the course of mental illnesses and potentially lead to new, effective forms of preventive treatment.

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6 Lactation: A Physiological Model of Stress Hyporesponsiveness of the Neuroendocrine System

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In response to physical or psychological stimuli perceived as stressful, an individual will exhibit acute changes in neuroendocrine function. These changes have important protective homeostatic properties and hyper- or hyporesponsiveness of neuroendocrine activity may, in certain circumstances, be detrimental to an individual's long-term health. For this reason, the mechanisms that determine the magnitude of the neuroendocrine response to stress are of major importance to medical research. Experimental studies of the variability of stress responses have frequently exploited the differences that occur between rat strains, such as Fischer vs. Lewis, Roman high- vs. low-avoidance, and Sprague-Dawley vs. Wistar (Gentsch *et al.*, 1982; Sternberg *et al.*, 1989; Rivest and Rivier, 1994; Harbuz *et al.*, 1994), or differences that are imprinted during neonatal development, as a result either of sexual differentiation (Carter *et al.*, 1986, 1988) or of early life experiences (Meaney *et al.*, 1994). In addition to this variability between individuals, stress responsiveness may also vary greatly during an individual's life time, such as the stress hyporesponsive state that occurs during the neonatal period (Walker *et al.*, 1991) and the altered responses seen at different times in the female reproductive cycle (Viau and Meaney, 1991). In this article we review the status of studies on stress responses during lactation.

During pregnancy and lactation many changes occur in the neuroendocrinology, physiology and behaviour of the female rat. These adaptive changes are important to ensure that the physiology and behaviour of the animal is appropriate for the successful care and growth of the offspring. A major change in neuroendocrine activity that is seen during lactation is the suppression of the neuroendocrine responses to acute stressful stimuli. This suppression may have adaptive significance in blocking inappropriate defence behaviour and allowing the mother to conserve metabolic energy. The fact that normal responses return soon after weaning indicates that lactation represents an easily inducible and fully reversible model of a stress-hypo responsive state.

CHARACTERISTICS OF THE LACTATION INDUCED STRESS-HYPORESPONSIVE STATE

(1) Effect of Lactation on Different Stress Hormones

Hypothalamo-Pituitary-Adrenal (HPA) Axis

Lactation is associated with changes in the mean levels and circadian patterns of corticosterone (CORT) secretion. Although the precise changes are controversial and great variability has been seen between studies, the most consistent observations appear to be a decrease in the evening peak of CORT levels (Stern *et al.*, 1973; Atkinson and Waddell, 1995) and a concomitant elevation of the morning nadir compared to virgin animals (Stern *et al.*, 1973; Walker *et al.*, 1992). Again, this is associated with an increase in the morning nadir seen in the virgin animal (Walker *et al.*, 1992; Atkinson and Waddell, 1995). Little is known of CRF release in the lactating rat, but CRF mRNA expression within the hypothalamus appears to be normal or reduced during lactation (Lightman and Young, 1989a; Fischer *et al.*, 1995; Windle *et al.*, 1996a), probably depending on the duration of lactation.

Much of our recent data has been obtained from rats chronically implanted with jugular catheters and sampled either remotely from outside the cage, or using a computer-controlled automated microsampling system in which the experimenter remains outside the room (Clark *et al.*, 1986). Throughout the sampling procedure the animal can behave normally and interactions between dam and pups are not interrupted. Under these conditions it is apparent that basal CORT levels in virgin animals are markedly pulsatile (Windle *et al.*, 1995). These pulses occur across the whole circadian cycle on top of a basal level that peaks in the evening. The fact that large pulses even occur during the morning nadir provide a ready explanation why data obtained from single time points shows a skewed distribution (e.g. Walker *et al.*, 1992). However, despite this pulsatile release in both virgin and lactating rats, we have been unable to detect any difference in the early morning levels of CORT or ACTH (Windle *et al.*, 1996a).

In contrast to the variable reports on basal activity, responses of the HPA axis to acute stress are consistently suppressed in lactating animals. Both the CORT response to electric footshock (Thoman *et al.*, 1970) and the ACTH response to a Porsolt forced swim stress (Walker *et al.*, 1995) are significantly attenuated during lactation, and ether-induced secretion of CORT and ACTH are diminished at all points throughout the circadian cycle (Thoman *et al.*, 1970; Stern *et al.*, 1973; Walker *et al.*, 1992). Furthermore, although the CORT levels are not affected, the normal increase in corticotropin-releasing factor (CRF) mRNA seen in the PVN following ip injection of hypertonic NaCl is abolished during lactation (Lightman and Young, 1989a). The HPA axis is not, however, prevented from responding to afferent stimuli, since the suckling stimulus itself induces a large release of both ACTH and CORT (Walker *et al.*, 1992). Our recent studies have employed two further psychological stresses to characterise the hyporesponsive state of the HPA axis. These have shown that: (1) the stimulated levels of CORT measured in lactating rats after a 30-min restraint stress are less than half those of virgin controls (da Costa *et al.*, 1996a); and that (2) lactating animals show no increase in either

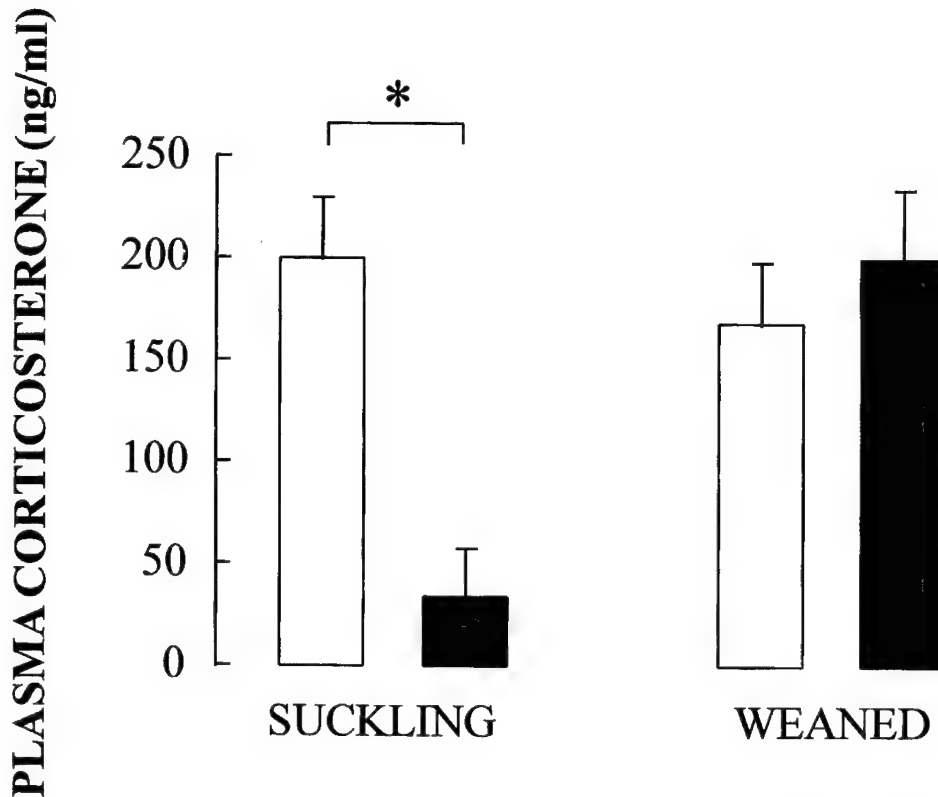


Figure 6.1 Peak plasma corticosterone concentrations following white noise stress (114 dB for 10 min) in virgin (open bars) or lactating rats (filled bars). The response to noise was measured on day 7–10 of lactation with the pups continuously suckling. The pups were then weaned and the response in the virgin (open bars) and post-lactating rats (filled bars) measured again. * $P < 0.05$ Student's *t*-test.

ACTH or CORT in response to an inescapable noise (10 minutes white noise for 114 dB) stress (Figure 6.1; Windle *et al.*, 1996a). The latter stimulus in conjunction with the automated microsampling system discussed above has been particularly useful by permitting, for the first time, the study of serial samples taken from freely behaving rats that have not been separated from their litters during stress. Furthermore, it is a relatively mild stress which acts as a good discriminator of a variable stress response.

Prolactin

Similar to the situation for the HPA axis, prolactin levels also rise following both physical and psychological stresses (e.g., restraint, ether stress, formalin injection), but none of these stimuli are effective in increasing prolactin levels in the lactating rat (Higuchi *et al.*, 1989, 1992; Walker *et al.*, 1992; Banky *et al.*, 1994). Indeed, it is more common to see a decline in the high levels induced by suckling due to

disruption of nursing (Walker *et al.*, 1992; Banky *et al.*, 1994; Windle *et al.*, 1996a). However, the high prolactin levels *per se* do not appear to prevent the response, since even when basal levels are made identical by separation of the mother from her pups, a 10-minute restraint will cause a large increase in prolactin in virgin but not lactating rats (Higuchi *et al.*, 1989). Further consideration of the effect of hyperprolactinaemia is given below.

Oxytocin

In contrast to male rats, female rats normally exhibit an increase in plasma oxytocin in response to immobilisation (Carter *et al.*, 1986, 1988; Higuchi *et al.*, 1986, 1988, 1990; Carter and Lightman, 1987; Miyata *et al.*, 1995). In virgin rats oxytocin levels are elevated within 1 minute (Carter and Lightman, 1987) and can remain elevated even after 3 hours (Miyata *et al.*, 1995). During lactation a major reorganisation of the patterns of oxytocin secretion occur, such that the magnocellular neurosecretory neurones begin to display periodic reflex bursting which produces pulsatile hormone secretion that is appropriate for its contractile action on the uterus and mammary gland. Coincident with this reorganisation the restraint-induced release of oxytocin is greatly diminished (Carter and Lightman, 1987; Higuchi *et al.*, 1988). On the basis of electrophysiological recordings and hormone measurements following stimulation with iv cholecystokinin-8 (a stimulus to oxytocin secretion), it has been suggested that this reduced response of lactating rats simply arises from a change in the releasable hormone pool, rather than a change in neuronal excitability (Higuchi *et al.*, 1991). However, while the reduced content may contribute to a reduction in the magnitude of responses, it is not clear that this entirely accounts for the loss of afferent activation by stressful stimuli. Our own quantitative analysis of restraint-induced *c-fos* mRNA expression in the magnocellular division of the PVN, indicates no difference between lactating and virgin animals (da Costa *et al.*, 1996a). However, this study did not identify specific subpopulations of neurones. It is not possible to determine whether stressful stimuli increase the steady-state levels of oxytocin mRNA in magnocellular neurones, as the high copy number masks any response (Lightman and Young, 1989b), and analysis of primary transcripts has not been performed.

One thing that is apparent from the data above is that the hormones normally associated with a neuroendocrine response to stress (i.e., ACTH, CORT, oxytocin, prolactin), are the very same hormones which are released in response to suckling and are important for the production and secretion of milk. Thus, it appears that the neuroendocrine axes responsible for the release of these hormones have been subsumed for this alternative purpose, but whether a single coordinating factor is responsible for determining these changes in function is unclear.

(2) Induction and Reversibility of the Hyporesponsive State

The precise time at which the stress hyporesponsive state appears has not yet been defined. However, recent data suggest that responses to stress are already reduced during late pregnancy, indicating that induction requires neither parturition nor the

suckling stimulus. Restraint-induced *CORT* secretion and *c-fos* mRNA expression in the PVN are reduced by late pregnancy (days 19–21; da Costa *et al.*, 1996a), and the HPA response to the stress of being placed on an elevated plus maze is attenuated on days 18 and 22 of gestation, although the response is normal on day 15 (Douglas *et al.*, 1996). This suggests a temporal window for induction in the latter half of gestation. Furthermore, beyond this induction period, the degree of suppression appears to increase during the course of lactation, in that ACTH responses to swim stress are significantly smaller in rats during late lactation (days 17–19 postpartum) compared to midlactation (days 8–10 postpartum) (Walker *et al.*, 1995).

Although basal *CORT* levels of the lactating rat rapidly return to normal after weaning (Stern *et al.*, 1973; Walker *et al.*, 1992; Fischer *et al.*, 1995), ether-induced *CORT* release is still suppressed 24 hours after removal of the litter (Stern *et al.*, 1973). However, in our experiments there is a restoration of a *CORT* response to noise stress 72 hours after removal of the litter from animals in midlactation (days 8–10; Figure 6.1). Likewise, the accumulation of CRF and enkephalin mRNAs in the PVN after ip hypertonic NaCl is still absent 18 hours after weaning, but is fully restored 48–72 hours after weaning animals at day 5 of lactation (Lightman and Young, 1989a). Interestingly, for animals in late lactation (days 15–17), 72 hours separation appears to be insufficient for full restoration of responses (Windle, Dallman, Lightman and Ingram, unpublished data), which may again indicate a gradually increasing degree of suppression. Moreover, the prolactin response to 10 minutes restraint takes 8 days to be fully restored after weaning on day 7 of lactation (Higuchi *et al.*, 1992). This difference in the time course of restoration of the HPA and prolactin axes strongly suggests that there are separate mechanisms maintaining the suppression of the two neuroendocrine axes.

NEUROANATOMICAL SUBSTRATES FOR THE HYPORESPONSIVE STATE

In addition to studying the lactating rat in respect of changes to plasma hormone levels, it is possible to study changes in neuroendocrine activity in terms of the expression of immediate (*c-fos*, NGF1-B) or late (CRF, AVP) genes using *in situ* hybridization histochemistry. This has the added advantage of being able to determine specific areas of the neuroaxis that may participate in generating the hyporesponsive state. Studies using a wide variety of stressors (e.g., restraint, ip hypertonic saline, ether vapour, footshock) have shown that the PVN may be activated by stress, as indicated by the increase in immediate early gene, CRF, AVP, and enkephalin mRNAs (Lightman and Young, 1987, 1988, 1989b; Harbuz and Lightman, 1989, 1992; Imaki *et al.*, 1993; Arnold *et al.*, 1992; Chan *et al.*, 1993; Rivest and Rivier, 1994; Cullinan *et al.*, 1995; Kovacs and Sawchenko, 1996). Although the pathways that transduce stimuli to the neuroendocrine hypothalamus remain poorly defined, studies using immediate-early genes have allowed a more extensive definition of the structures involved (e.g., Arnold *et al.*, 1992; Cullinan *et al.*, 1995). Common structures activated by different stressors include the brainstem (locus coeruleus, dorsal vagal complex), limbic system (amygdala, lateral septum,

hippocampus, cingulate and pyriform cortices), and diencephalon (arcuate nucleus, PVN, preoptic area, mammillary nuclei). By comparing these regional patterns of gene expression in lactating and nonlactating rats, we have been trying to identify areas that show reduced responses. The absence of a neuroendocrine response correlates well with a lack of activation in the PVN: CRF and enkephalin mRNA responses to ip NaCl are reduced in the PVN of lactating animals (Lightman and Young, 1989a), and *c-fos* mRNA expression following restraint (da Costa *et al.*, 1996a) is also attenuated during lactation. This confirms that afferent pathways either do not reach or fail to activate the nucleus. In extrahypothalamic areas, restraint stress will lead to *c-fos* mRNA expression in a wide range of areas. However, in the cingulate cortex, medial amygdaloid nucleus, and the ventral part of the lateral septum, the activation shown by lactating rats is significantly down-regulated (da Costa *et al.*, 1996a). This suggests that these structures may contribute to the reduced neuroendocrine responses of the lactating rat, possibly by affecting afferent pathways; current studies are addressing the nature of this contribution.

POSSIBLE MECHANISMS REGULATING STRESS HYPORESPONSIVENESS

The fact that stress fails to activate immediate-early genes in the PVN during lactation suggests that the mechanisms underlying the hyporesponsive state involve the afferent signals reaching the PVN, rather than the output from these neurosecretory neurones. Many transmitter systems are altered during pregnancy and lactation, and several are candidates for modulating stress-induced neuroendocrine responses. Furthermore, the dramatically altered hormone levels provide the potential for feedback regulation, particularly by steroid hormones. In the following section we consider the possible mechanisms underlying the stress hyporesponsiveness of lactating rats.

(1) Role of Gonadal and Adrenal Steroid Hormones

In the rat, steroid hormone levels change in a dynamic manner during the later stages of pregnancy and at parturition (Garland *et al.*, 1987; Atkinson and Waddell, 1995), and these may serve to trigger the beginning of the hyporesponsive period. To our knowledge there have been no studies that have directly addressed whether these changes in gonadal steroids can affect later stress responses. However, in virgin animals, neither ovariectomy nor ovariectomy with replacement of oestradiol and progesterone modifies immobilisation-induced release of oxytocin (Carter and Lightman, 1987), suggesting that these conditions are unable to induce a hyporesponsive state. Furthermore, the failure of lactating rats to respond to stress appears not to be due to increased corticosteroid feedback. The reasons for this conclusion include the observations that: (i) adrenalectomy does not restore the ACTH response to forced swim stress in lactating animals (Walker *et al.*, 1995); (ii) while lactation is associated with a complete suppression of the CRF mRNA response to the stress of ip hypertonic NaCl (Lightman and Young, 1989a) but only slightly

modified basal CORT levels (see above), even very high levels of exogenous glucocorticoids cannot completely block this response in male animals (Harbuz *et al.*, 1990); and (iii) adrenalectomy and replacement with constant levels of CORT show that corticosteroid negative feedback is intact and, after compensation for the differences in absolute levels of ACTH, the sensitivity to negative feedback is unaltered (Walker *et al.*, 1992). Furthermore, the presence of normal negative feedback has been shown by the parallel time course and magnitude of the CRF mRNA accumulation following adrenalectomy of virgin and lactating rats (Lightman and Young, 1989a). Therefore, although one cannot rule out the possibility that CORTs are involved in the induction but not the subsequent maintenance of the hyporesponsive state, neither the changes in plasma levels of CORT nor changes in sensitivity appear to be involved in this state. It is, therefore, more likely that a modulation of circuits transducing the stress signal to neuroendocrine centres in the hypothalamus is involved.

(2) Plasticity of Catecholaminergic Systems

The extensive literature on the catecholaminergic innervation of the PVN and the actions of the catecholamines on CRF release have been reviewed elsewhere (Plotsky *et al.*, 1989; Al-Damluji *et al.*, 1988). It is generally accepted that noradrenergic and adrenergic pathways are important components in the transmission of peripherally perceived stress stimuli from the brainstem, predominantly via the ventral noradrenergic bundle to the hypothalamus. Most, but not all, reports suggest a positive role of catecholamines on the release of CRF both *in vivo* and *in vitro* (Al-Damluji, 1988; Szafarczyk *et al.*, 1988; Plotsky *et al.*, 1989). This may represent either a direct effect on the PVN or an indirect effect via pathways involving other limbic areas. The stimulatory effect of the catecholamines is mediated, at least in part, by α_1 adrenoceptors and can be blocked by the α_1 antagonist prazosin (Szafarczyk *et al.*, 1987; Itol *et al.*, 1994). ICV injection of the α_1 agonist methoxamine in the virgin animal results in an acute elevation of plasma oxytocin and ACTH (Patel *et al.*, 1993) and a long-lasting increase in plasma CORT levels together with a doubling of CRF mRNA levels within the PVN, 2 hours after injection (Ingram *et al.*, 1996). In contrast, similar injections into lactating rats have only a small and transient effect on plasma CORT, ACTH, and oxytocin, and no effect on CRF mRNA levels. We have investigated whether this difference was due to a reduced sensitivity of PVN neurones to this agonist by *in vitro* extracellular electrophysiological recording. PVN neurones from virgin animals showed an increase in firing rate in response to both 10^{-5} and 10^{-4} M methoxamine, but neurones from lactating animals only responded to the higher dose (Ingram *et al.*, 1996). This indicates that a reduced sensitivity to noradrenergic afferents may be one of the mechanisms underlying the hyporesponsive state seen during lactation.

It is known that oestradiol can upregulate the hypothalamic α_1 adrenoceptor population (Etgen *et al.*, 1992). Therefore the steroidal changes that occur during late pregnancy and lactation could influence the catecholaminergic activation of the PVN. To test this we have compared the effect of icv injection of methoxamine in virgin and ovariectomized rats treated with oil, oestradiol, or progesterone implants.

This showed that, although there was a slight reduction in the CORT response following ovariectomy, the HPA response was not abolished as it was in the lactating rats. Furthermore, none of the steroid treatments have any significant effect on the response (Kunanadam, Windle, Lightman and Ingram, unpublished data). Therefore, although a gonadal steroid interaction with catecholaminergic transmission may contribute to the hyporesponsive state, it does not appear to be the primary cause.

(3) Lactational Hyperprolactinaemia

High levels of prolactin have been suggested to play a role in the suppression of hypothalamo-pituitary-ovarian axis during lactation and may play a role in controlling other axes. Prolactin binding sites have been detected in the limbic system and, in addition to the *de novo* synthesis of prolactin within the brain, specific uptake mechanisms exist to transfer the high circulating levels across the blood-brain barrier. Hyperprolactinaemia induced over six days by the dopamine antagonists domperidone or haloperidol, or by injection of exogenous ovine prolactin, all reduce immobilisation-induced release of oxytocin in virgin female rats (Carter and Lightman, 1987). Furthermore, the effect of domperidone can be increased by simultaneous steroid treatment involving ovariectomy with oestradiol and progesterone replacement, under which conditions immobilisation no longer induces oxytocin release (Carter and Lightman, 1987). However, evidence against prolactin being the regulatory factor in lactational hyporesponsiveness include the following: (i) the hyporesponsive period begins in late pregnancy before the prolactin surge on day 22; (ii) although restoration of basal prolactin levels rapidly follows cessation of suckling (Banky *et al.*, 1994), the evidence given above suggests that stress-induced responses continue to be suppressed for up to 8 days; (iii) unlike lactation, domperidone does not affect the induction of CRF and enkephalin mRNA transcription in the PVN by hypertonic saline (Lightman and Young, 1989b); and (iv) treatment of ovariectomized rats with oestradiol greatly increases circulating prolactin levels but does not block the methoxamine-induced release of CORT (Kunanadam, Windle, Lightman and Ingram, unpublished data), a stress-related response which is blocked during lactation (see above).

(4) Coordinating Role of Oxytocin

During lactation the synthesis and release of central oxytocin is increased, and the density of oxytocin binding sites shows region-specific upregulation (Insel, 1990). This has been proposed to play a major role in: (i) the anatomical reorganisation of the magnocellular neurones of the supraoptic and paraventricular nuclei during lactation; (ii) the induction of maternal behaviour; and (iii) the feedback control of reflex release of oxytocin (Wakerley *et al.*, 1995; Ingram *et al.*, 1995a,b). However, this coordinating function may also extend to regulation of the stress axis. An indication that oxytocin may alter stress responses has come from the fact that peripheral injection of high-dose oxytocin has an anxiolytic effect on open-field behaviour (Uvnäs-Moberg *et al.*, 1994), and direct injection of oxytocin or vasopressin

into the amygdala attenuates conditioned fear responses (bradycardia and immobility) in Roman low-avoidance rats (Roozendaal *et al.*, 1992). To examine whether central oxytocin is capable of suppressing neuroendocrine responses to stress, we have studied virgin rats (ovariectomized with oestradiol replacement) implanted with Alzet osmotic minipumps connected to a cannula in order to deliver oxytocin into the lateral cerebral ventricle over a period of 7 days (Windle *et al.*, 1996b). When tested for their response to noise stress after 5 days, these animals showed a highly attenuated response compared to saline-infused controls. However, when tested 8 days postinfusion, the responses were the same as the controls (Figure 6.2). Furthermore, tests for anxiety using an elevated plus maze, showed that oxytocin-treated animals exhibited significantly more entries onto the open arms compared to controls (Windle *et al.*, 1996b). Thus, oxytocin had both an anxiolytic effect and reduced the neuroendocrine responses to stress. This dose-related effect, was observed even at the lowest dose tested (1 ng/h); no comparable effect was seen with

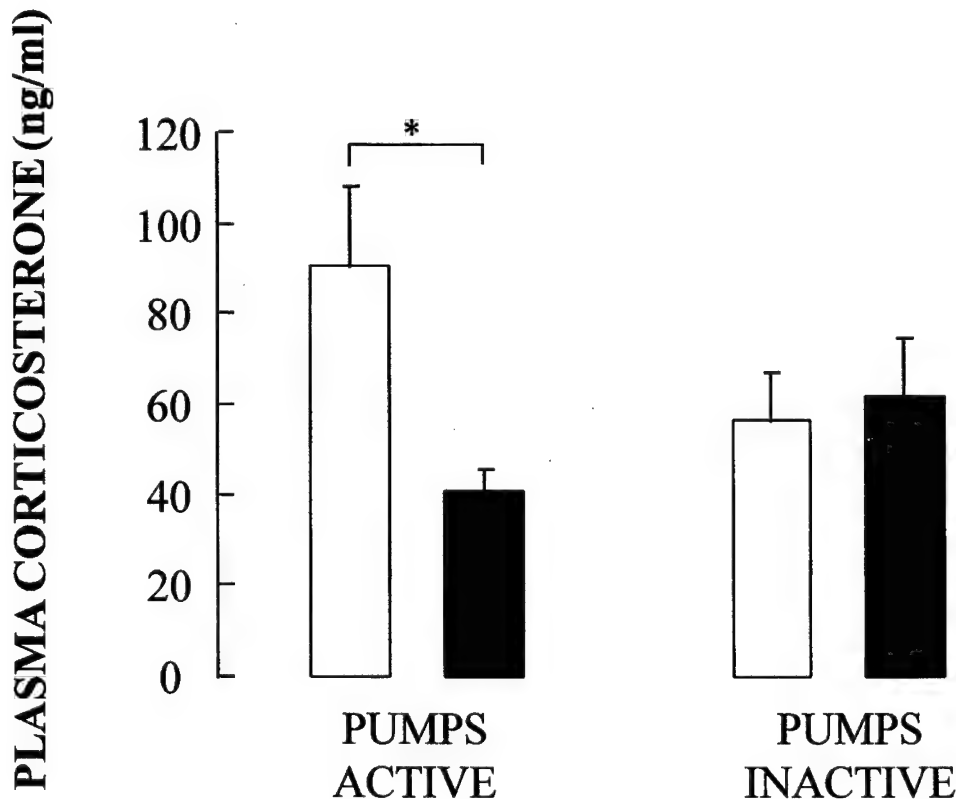


Figure 6.2 Peak plasma corticosterone concentrations following white noise stress (114 dB for 10 min) in ovariectomized, oestradiol-treated rats. Animals were fitted with 7 day osmotic minipumps filled with either isotonic saline (open bars) or 200 µg/ml oxytocin (filled bars). The response to noise was measured 5 days after minipump implantation when the pumps were active and 72 h later when the pumps had become inactive. * $P < 0.05$ Student's *t*-test.

vasopressin. Although we have not yet identified this site of action, it is interesting to note that the areas that displayed modified immediate-early gene responses to stress (i.e., cingulate cortex, lateral septum, amygdala) all express oxytocin binding sites. Electrophysiological recordings have shown that the ventrolateral septum shows marked modulation of responses to oxytocin during the peripartum period (Ingram *et al.*, 1995b; Wakerley *et al.*, 1995). Thus, the possibility that the anxiolytic effect of oxytocin underlies the hyporesponsive state observed during lactation is an attractive idea that is currently under investigation.

(5) Down-regulation of the Stress-mediating Action of CRF

CRF appears to play a pivotal role in coordinating the physiological, behavioural, and neuroendocrine responses to stress. Infusions of CRF evoked activation of the PVN (Arnold *et al.*, 1992; Imaki *et al.*, 1993) and neuroendocrine responses similar to those seen during stress; moreover, CRF antagonists blocked the stress-induced activation of the PVN (Imaki *et al.*, 1995). Since central CRF pathways may be part of a system coordinating central responses to stress (Dunn and Berridge, 1990), changes in the activity of this system might lead to a hyporesponsive state. Indeed, this has been suggested to be the reason for the stress hyporesponsiveness of the Lewis rat (Sternberg *et al.*, 1989). To test whether postreceptor modulation of CRF action occurred during lactation, we tested the effect of icv injection of 5 µg CRF on the release of oxytocin in lactating and nonlactating rats. The data showed that CRF evoked release of oxytocin in virgin female rats but had no effect in lactating rats, even when administered at a tenfold higher dose (Patel *et al.*, 1991). To examine whether this relates to a region-specific downregulation of responses to CRF, we examined the pattern of immediate-early gene mRNA expression following central injection of CRF. This showed that CRF evoked increased expression of NGF1-B and *c-fos* mRNAs in the lactating rat to the same extent as in virgin animals in some regions (e.g., dentate gyrus). However, the responses observed in the CA3 region of the hippocampus, lateral septum, amygdala, and PVN were all significantly reduced (da Costa *et al.*, 1996b). These data suggest that, similar to the response to restraint, specific areas of the limbic system show attenuated activation during lactation, and these may comprise a circuit which contributes to regulation of the responses to stress.

SUMMARY AND CONCLUSIONS

From a period beginning in the latter stages of pregnancy and continuing throughout lactation, the female rat displays a marked reduction in the neuroendocrine response to stress. This is a generalised hyporesponsiveness, as it is independent of the nature of the stress employed and simultaneously affects several neuroendocrine axes (HPA responses, oxytocin, and prolactin). Although the induction of this state does not require the suckling of the young, premature weaning leads to a restoration of normal responses. The mechanisms underlying the hyporesponsiveness appear not to involve changes in corticosteroid feedback or simple effects of gonadal steroids, but modulation of the central transmitter activities of noradrenaline and CRF appear to

play a role. Modulation of these and other (e.g., opioid) transmitter pathways not considered in this review may contribute to the reduced afferent activation of the PVN and of limbic structures that may comprise a circuit that determines the magnitude of a response to stress. Whether this circuit is subject to control by central oxytocin through an endogenous anxiolytic action is the focus of continued research.

These studies clearly demonstrate that the psychoneuroendocrine system is not static but shows dynamic adaptive responses which are appropriate for particular physiological states. Although it is not clear why lactation should be associated with reduced responses to stress or whether dysregulation of this change is detrimental either to the dam or her litter, this condition does afford an excellent model for studies of stress hyporesponsiveness. Continued studies of this model may finally lead to the elucidation of the neural systems which determine the magnitude of neuroendocrine responses to stress.

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7 Limbic Mechanisms of Anxiety and Stress

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Most workers in the field of stress would concede that the concept is imprecise. This problem is readily apparent in a relatively recent definition of stress by the progenitor of the concept, Hans Selye: "...the nonspecific...result of any demand upon the body, be the effect mental or somatic" (Selye, 1982). Despite being an imprecise definition, the strong implication is that many kinds of input can lead to a common set of bodily reactions that we normally refer to as the stress reaction. Thus a common stress response may be seen to disease, pain, fear, and anxiety. Indeed anxiety is very likely the most significant psychological variable giving rise to the stress reaction.

Anxiety is a major source of mental distress in humans and animals. There have been a number of major advances in the understanding of the brain mechanisms mediating anxiety and its relation to the stress response, as well as the mechanism of action of a variety of drugs and pharmacological agents that affect anxiety and the stress reaction. The evidence indicates that there is considerable overlap and crosstalk between brain systems related to anxiety and the systems related to the stress reaction. Therefore, in order to understand the relation between anxiety and stress it is important to understand how the brain mechanisms of anxiety are organized and how they interact with the systems involved in the stress response. In this paper we will examine the role of two major limbic system structures, the lateral septum and the amygdala.

In the early part of this century the Swiss physiologist Hess (1954) described a mechanism for the mediation of emotion that has merit even today, especially with regard to the mechanisms that can modulate the stress response. Hess suggested that the diencephalon could be divided into two general regions. One region he referred to as ergotropic, which generally mediated sympathetic autonomic responses and was related to behavioral arousal and strong emotions such as fear and rage. The second region he referred to as trophotropic. This region generally mediated parasympathetic autonomic responses and behaviorally was related to relaxation and

lowered emotionality that, in terms of degree, Hess referred to as adynamia, atony, and ultimately, sleep. The presumption was that these two mechanisms were reciprocally related and mutually inhibitory. Presumably, one's emotional state at any given moment would depend upon the autonomic balance between the two mechanisms (Gellhorn and Loofbourrow, 1963).

We suggest here that structures in the limbic system bear a similar ergotropic/trophotropic relation to each other in the mediation of anxiety. In particular, we argue that the amygdala and the lateral septum have reciprocal roles in the modulation of anxiety and anxiety-mediated stress. Moreover, the interaction of these structures plays an important role in the mechanism of action of anxiolytic drugs. In general, the amygdala appears to play an important role in the expression of fear while the lateral septum appears to have an anxiety-relief function. There is a striking parallel between the reciprocal functions of these structures in fear and anxiety, as well as their functions in the stress response. The evidence suggests that the amygdala is involved in the expression of the somatic sequelae of stress, whereas the lateral septum may mitigate the effects of stress, possibly by acting through the amygdala.

Recent evidence from our laboratory provides support for the idea that the lateral septum plays an active role both in the relief of fear and the mitigation of the stress reaction. These experiments looked at stimulation and lesion effects on some well established behavioral paradigms, known to be related to anxiety and/or stress. These paradigms included the water-lick suppression conflict procedure as a test for anxiety and the production of stomach ulcers by the cold-immobilization stress procedure.

CONFLICT

The conflict test we used was a modified version of the Vogel water-lick suppression test (Vogel *et al.*, 1971). It entailed the use of rats deprived daily of water for 23.5 hours. They were run in a clear Plexiglas chamber with access to a water tube. A 2-minute unsignaled, unpunished period of licking was followed by a 2-minute signaled punished period, during which every 20th lick was accompanied by a mild electric shock to the chamber grids. These two periods cycled through twice to establish stimulus control over the behavior. Analysis was always done only on the first two 2-minute periods, as those were discerned empirically to yield the most stable and reliable data. Normally, rats run in this procedure, with shock levels adjusted individually, reach a steady baseline behavior within two weeks of training, in which they make a high number of licks during the unpunished period and suppress their licking to approximately 25% of that rate during the signaled punished period.

Electrical stimulation of the lateral septal nucleus during the aforementioned conflict procedure resulted in a significant increase in the number of licks during the punished period — an 'anxiolytic' effect (Yadin *et al.*, 1993). The degree of release during the punished period with lateral septal stimulation was similar to that seen after administration of a known anxiolytic agent, the benzodiazepine

chlordiazepoxide, at a dose of 10 mg/kg. Intraseptal administration of the benzodiazepines chlordiazepoxide (60 µg) or midazolam (30 µg) also produced a significant increase in the number of punished licks (Grishkat, 1991). In contrast, lesions of the lateral septal nucleus produced an 'anxiogenic' effect — a further significant decrease in the number of licks during the punished period.

IMMOBILIZATION

A procedure used in our laboratory to test the role of the lateral septum in the stress reaction was the gastric ulcer-producing method of cold-immobilization stress (Yadin and Thomas, 1996). It entailed the restraining of 24-hour food-deprived rats for 3 hours in a cold room, at 4°C. Throughout the immobilization period one rat in each pair received low current trains of pulsed electrical stimulation of the lateral septal nucleus, while its control counterpart received none. After the immobilization session was completed, rats were sacrificed and their stomachs removed, cut along their greater curvature and gastric ulcers were quantified.

The control animals' stomachs contained a large number of gastric ulcers after 3 hours of cold immobilization, while their septally-stimulated partners displayed significantly fewer lesions, and in many cases, were entirely devoid of ulcers. Lateral septal stimulation appeared to drastically diminish the number and severity of the gastric ulcers produced during a stress session.

These data on the effect of septal stimulation on anxiolysis in the conflict situation and on ulcer formation in the cold-immobilization procedure are in direct contrast with what is seen in the amygdala. Electrical stimulation of the central nucleus of the amygdala is anxiogenic, as tested in the startle paradigm (Rosen and Davis, 1988). Stimulation of the same region of the amygdala induces stomach ulceration similar to that induced by immobilization stress (Henke, 1985; Henke and Sullivan, 1985).

Septal/amygdala reciprocity in the stress response may also be seen in the effect of stimulation upon corticosteroid release. Stimulation of the central nucleus of the amygdala promotes corticosteroid release (Matheson *et al.*, 1971), whereas, consistent with an antistress role for the lateral septum, electrical stimulation of this region reduces corticosteroid output (Saphier and Feldman, 1987).

The experiments described above are consistent with a considerable literature, based upon stimulation, single unit recording and lesioning experiments, pointing to reciprocal functions of the amygdala and the lateral septum. We will describe briefly each of these sources of data.

STIMULATION EXPERIMENTS

A number of experiments have pointed to a fear- or anxiety-inducing effect of stimulation of the amygdala. Electrical stimulation of the amygdala produces a variety of sympathetic autonomic responses characteristic of that seen in anxiety and stress (such as changes in heart rate, respiration, pupillary responses) (Applegate *et al.*, 1983; Gloor, 1960). Behaviorally, stimulation of the amygdala results in general

Table 7.1 Effects of electrical stimulation of the amygdala and lateral septum upon standard measures of fear and stress in animals

<i>Measure of fear or stress</i>	<i>Amygdala stimulation</i>	<i>Septal stimulation</i>
Heart rate	↑	↓
Salivation	?	↑
Respiration change	↑	↓
Scanning and vigilance	↑	↓
Startle	↑	↓
Urination	↑	?
Defecation	↑	?
Grooming	↑	After effect (see text)
Freezing	Increased freezing	Relaxation (see text)
Stomach ulcers	Ulcers increased	Ulcers prevented
Corticosteroid release	↑	↓

defense reactions including freezing, increased alertness, and, in cats, vigorous escape and attack behaviors (Kaada, 1951). In the rat, electrical stimulation of a number of nuclei in the amygdala, principally the central nucleus, potentiates the acoustic startle response in a manner similar to the potentiation produced by a conditioned fear stimulus (Rosen and Davis, 1988).

By contrast, there is an extensive literature (reviewed by Thomas, 1988) pointing to the fact that electrical stimulation of the septum yields both somatomotor inhibition, typically in the form of a behavioral arrest reaction, and visceromotor inhibition. In addition, in early work in our laboratory (Beagley, 1972) we observed in cats that stimulation resulted in a general calming effect upon the animal. If the animals were showing signs of distress, such as mewling and vigorous struggling against a restraint, such behavior was greatly suppressed by septal stimulation (Table 7.1). This suppression was almost invariably followed by a postinhibitory rebound increase in distress vocalization and struggling. In the rat this rebound is seen as a poststimulation increase in grooming behavior (Table 7.1). A summary of the opposing effects of stimulation of the amygdala and the lateral septum is presented in Table 7.1.

SINGLE-UNIT RECORDING

The role of the septum in the relief of fear has been supported by multiple- and single-unit recording in several regions of the septal area. If the septum is involved in the relief of fear it should be possible to record unit activity in the region correlated with such relief. There is considerable evidence that in Pavlovian conditioning a stimulus explicitly unpaired with an aversive unconditioned stimulus (US) becomes a conditioned inhibitor of fear and gains the capacity to actively relieve fear (Rescorla, 1969; Weisman and Litner, 1972). If such is the case then the presentation of a conditioned inhibitor of fear should be reflected in increased unit activity in fear-inhibitory regions such as the lateral septum. We carried out a series of multiple unit recording experiments in which rats were subjected to a Pavlovian differential conditioning paradigm, with light or tone as the conditioned stimulus (CS) and

footshock as the US (Thomas and Yadin, 1980; Yadin and Thomas, 1981). One CS (CS+) was paired with the aversive US and the other CS (CS-) was presented in the absence of the US. In such a paradigm the CS+ becomes a conditioned excitator of fear and the CS- a conditioned inhibitor of fear.

Increased multiple unit activity above and beyond baseline activity was seen in the septal region in the presence of CS-, whereas multiple unit activity was suppressed in the presence of the CS+. Increased unit activity was also seen at the termination of the shock US and at the termination of a tail pinch. Evidence from the learning literature suggests that the termination of an aversive stimulus is associated with the relief of fear (e.g., Denny, 1971; Konorski, 1972). Thus, in our experiments we had a variety of stimuli known to be associated with fear relief, the presentation of a conditioned inhibitor and the termination of two kinds of aversive stimulus, footshock and a tailpinch. Increased unit activity in the lateral septum was seen in all these cases. This effect was quite specific and was not seen in other brain regions, nor in the septum to a CS- when the US was appetitive rather than aversive. More recent single-unit studies (Thomas and Yadin, 1987; Thomas *et al.*, 1991) have generally verified the multiple-unit data.

Since cells in the septum respond in a consistent manner to stimuli associated with fear relief, it might be expected that the same cells would respond similarly to pharmacological agents that affect fear. Specifically, anxiolytic agents such as the benzodiazepines should increase the firing rate of cells in the lateral septum in a manner similar to a conditioned inhibitor of fear.

A recent dissertation in our laboratory systematically explored the effect of benzodiazepine administration upon unit activity in several septal regions. The research was carried out in animals that had been subjected to Pavlovian fear conditioning. In addition to measuring unit activity we were able to measure gross movement in the animals, both horizontal and vertical, using a video tracking system. Thus, we were able to correlate and directly compare overt movement and unit activity.

The data were very consistent: benzodiazepines affect cells in the lateral septum in a manner virtually identical to the effect of a conditioned inhibitor of fear. Thus, when chlordiazepoxide (5 mg/kg) was administered to rats that had been classically conditioned in a discrimination paradigm, there was an increase in baseline firing rates and an enhancement of the ability of a conditioned inhibitor of fear to increase unit activity. Chlordiazepoxide also blocked the suppression of unit activity normally seen to the conditioned excitator of fear (Strickland, 1993). Of considerable importance was the fact that there existed a clear dissociation between the changes in unit activity and the actual behavioral movement patterns associated with the conditioning paradigm. The changes in unit activity were not dependent upon the animal's overt behavior.

It is interesting that response patterns of single units in the amygdala appear to be the inverse of what is seen in the lateral septum. Thus, in the central nucleus of the amygdala cells show increased firing rates to a conditioned fear stimulus (Applegate *et al.*, 1983). The effect of a conditioned inhibitor of fear on unit firing in the amygdala has not been tested. Benzodiazepine administration decreases firing rates of units in the amygdala in the acute preparation (Chou and Wang, 1977).

Table 7.2 Response of single units in the amygdala and lateral septum to conditioned excitors and inhibitors of fear and to benzodiazepine anxiolytics

	<i>Amygdala</i>	<i>Septum</i>
Conditioned excitor of fear	↑	↓
Conditioned inhibitor of fear	?	↑
Response to benzodiazepines	↓	↑

Table 7.2 summarizes the comparison of unit-activity responses in the lateral septum and amygdala.

LESIONING EXPERIMENTS

The early observation by Brady and Nauta (1953) of a hyperexcitability syndrome in rats with septal lesions provides a striking contrast with the behavior seen in animals with amygdala lesions. The behaviors observed in septally-lesioned animals included increased aggression, vocalization, urination, defecation and an increased startle response. The behaviors seen were only temporary and tended to diminish dramatically, especially with handling, over a few days (Fried, 1973). Nevertheless, some less dramatic remnants of septal hyperemotionality remain. For instance, in the conflict experiment we found that lesions of the lateral septum result in anxiogenesis (Yadin *et al.*, 1993). This is manifest by added suppression of punished responding in the Vogel water-licking conflict test (Vogel *et al.*, 1971). Sparks and LeDoux (1995) have found recently that lesions of the lateral septum result in enhanced conditioning of fear to contextual cues.

It should be noted that there are some substantial inconsistencies in the effects of septal lesions and alternative interpretations of septal lesion effects. It is, therefore, necessary to digress, to deal with what is a somewhat complex and ambiguous problem.

Perhaps the most important alternative to the one we are positing is that proposed by Gray (1982). Gray points out the similarity between the effects of septal (and hippocampal) lesions and the effects of benzodiazepine anxiolytics in a number of paradigms, including passive avoidance. Both benzodiazepines and septal lesions result in slower acquisition of passive avoidance when a previously reinforced response is punished. In the standard passive avoidance task the animal must learn to inhibit a previously learned prepotent response. Benzodiazepines and septal lesions appear to interfere with the animal's ability to learn to inhibit prepotent responses. On the basis of these kinds of data, Gray proposes that the septum is part of a behavior inhibitory system that subserves anxiety and that lesions of the system act in the same anxiolytic manner as benzodiazepines. Consistent with this view is the finding that septal lesions produce what appears to be an anxiolytic effect when anxiety is measured in tasks such as the elevated plus-maze (Treit and Pesold, 1990).

Are septal lesions anxiolytic or anxiogenic? In several years of work in our laboratory with benzodiazepines and amygdala- and septal-lesioned animals, the

evidence most favors the notion that septal lesions are anxiogenic. Gross observation of the animal's behavior is convincing that the effect of benzodiazepines on the animal's general level of emotionality is entirely unlike what is seen in septal-lesioned animals. On the other hand, the behavioral effects of benzodiazepines appear very similar to the effects of amygdala lesions. Our data (Thomas and Snellman, 1996) suggest that while septal-lesioned animals tend to enter the open arms of the elevated plus-maze more than sham lesioned animals, they are nevertheless more anxious, hyperreactive, and show increased defecation on the open arms. An accumulation of evidence suggests that the lateral septum mediates the ability of stimuli to provide relief from fear. For instance, animals with septal lesions show an impairment in the capacity to inhibit fear in the presence of a CS- (Wagman, 1972). The inhibition of fear appears to serve as a reinforcement for much aversively motivated behavior and may well be the mechanism whereby an animal chooses the enclosed arms in the elevated plus-maze over the open arms. We suggest therefore that animals with septal lesions (and other anxiety-inducing lesions, such as those of the ventromedial nucleus of the hypothalamus) no longer benefit from the safety provided by the enclosed arms.

CONCLUSION

It seems clear that the brain possesses mechanisms that function to alleviate the stress placed upon the physiology of the body by anxiety. Compared to the systems involved in the generation of the stress response, relatively little is known about the circuitry of the anxiety/stress inhibition systems. The preponderance of the evidence, however, points to the lateral septum as a structure which serves in such a capacity. To understand the role of the septum, it will be necessary to gain some understanding of the outputs of the septum to structures involved in the stress response. Based upon a combination of electrophysiological data and lesioning data some tentative conclusions are possible. The septum has potent inhibitory influences over the paraventricular nucleus of the hypothalamus, a region central to the hypothalamic-pituitary-adrenal axis (Saphier and Feldman, 1987). It also inhibits brainstem structures associated with the perception of pain (Nasi and Thomas, 1982). Finally, lesion data suggest the pathway for septal influence over anxiety and stress is probably via the amygdala. In both the conflict paradigm and in fear-potentiated startle the anxiogenic effects of septal lesions are reversed by amygdala lesions with the effects of the amygdala lesions predominating (Grishkat, 1991; Melia *et al.*, 1992). Further research is currently underway to examine the reciprocal relationship between these two structures and their mediation of anxiety and stress. Of particular importance is to determine how these regions interact with downstream structures in the diencephalon and the brainstem to control their outputs in reaction to stress.

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8 Interaction Between Glucocorticoids and Neurotensin in Normal and Stress Responses

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Evidence supports a relationship between high levels of glucocorticoids (GCs) and depressive symptoms, suggesting the normalization of the hypothalamic–pituitary–adrenal (HPA) system activity for mood improvement (Nemeroff, 1992; Barden *et al.*, 1995). The action of antidepressants such as amitriptyline or desipramine could thus be related to the attenuation of adrenocorticotropin (ACTH) and corticoid hypersecretion via monoaminergic neurotransmission (Brady *et al.*, 1991; Reul *et al.*, 1993). Other mediators such as peptides, which have been shown to be involved in the neural control of the HPA axis, may also play an important role in keeping HPA axis activity at basal levels, in addition to classical antidepressants. Indeed, some of these neuropeptides appear to be altered in the cerebrospinal fluid of patients with major depression (Nemeroff, 1992).

Many different stressors are capable of inducing acute or chronic up-regulation of mRNAs encoding the main ACTH secretagogues such as corticotropin-releasing hormone (CRF) and arginine vasopressin (VP) in hypophysiotrophic neurons of the hypothalamus (Lightman and Young, 1988; Bartanusz *et al.*, 1993). Changes in CRF mRNA in the paraventricular nucleus of the hypothalamus (PVN), observed just prior to the onset of the dark period, have been shown to be independent of GCs levels (Kwak *et al.*, 1993). Therefore, neural inputs to the PVN are believed to convey diurnal or stress activation of parvocellular CRF/VP neurons leading to ACTH release. In support of this hypothesis, the stress-induced rise in CRF and VP transcripts in parvocellular neurons is inhibited after local hypothalamic administration of tetrodotoxin, a drug that blocks Na⁺ voltage-dependent synaptic transmission (Sawchenko *et al.*, 1993). During the last couple of years, much data have been accumulated suggesting that not only classical neurotransmitter substances such as monoamines, but also peptidergic afferents could specifically control CRF and VP release as well as gene expression in hypophysiotrophic neurons (Scaccianoce *et al.*, 1993; Suda *et al.*, 1993; Larsen *et al.*, 1993).

Among such neuropeptides, neurotensin (NT), a tridecapeptide (pGlu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu-OH) originally isolated from bovine hypothalami (Carraway and Leeman, 1973), was reported to increase plasma levels of ACTH and corticosterone (CORT) when administered intracerebroventricularly (icv) (Fuxe *et al.*, 1984; Gudelsky *et al.*, 1989; Nicot *et al.*, 1994). Pharmacological data suggest that this activation of the HPA axis might be mediated by an enhanced release of CRF from the median eminence to the portal vessels, since pretreatment of rats with the CRF antagonist, α -helical CRF, attenuates this effect (Rowe *et al.*, 1995). Direct neuroanatomical connections between NT and CRF neurons may underline the stimulatory effects of NT, since high densities of both NT terminals and binding sites have been observed in the parvocellular division of the PVN, where CRF-producing neurons are located (Sawchenko *et al.*, 1984; Emson *et al.*, 1985; Nicot *et al.*, 1994).

On the other hand, upregulation of NT mRNA was recently demonstrated in the PVN after ether or immobilization stress (Ceccatelli and Orazzo, 1993). Moreover, in bilaterally adrenalectomized rats or in normal animals, CORT implants induced an increase of NT mRNA in the periventricular nucleus of the hypothalamus (Watts and Sanchez-Watts, 1995; Nicot *et al.*, 1995). These results suggested that the activation of the HPA axis and GCs could modulate hypothalamic NT levels.

The present work sought to study the action of endogenous hypothalamic NT in the basal and stress-induced regulation of the HPA axis as well as in the regulation of basal plasma levels of VP and oxytocin in rats. Such study has been made possible by the recent development of nonpeptide NT receptor antagonists, able to block the NTergic neurotransmission by acting on the NT receptors (Gully *et al.*, 1993). On the other hand, by means of *in vitro* studies, we will try to investigate the regulation of hypothalamic NT neurons by GCs and elucidate their mechanisms of action.

INVOLVEMENT OF ENDOGENOUS NT IN THE ACTIVITY OF THE HPA AXIS

The onset of the NT-induced marked increases in plasma ACTH and CORT following icv administration has been shown to be rapid and last for several hours (Nicot *et al.*, 1994; Rowe *et al.*, 1995). Such effect was also parallel to a decrease in CRF content in the median eminence (Rowe *et al.*, 1995). Interestingly, it was recently observed that prior administration of the CRF-receptor antagonist, α -helical CRF, as well as lesions of the PVN blunted the stimulatory effects of NT on the release of ACTH and CORT (Rowe *et al.*, 1995). Although these findings suggest a role of NT via an effect on CRF neurons, they do not necessarily demonstrate that endogenous NT plays a role under physiological conditions and that the CRF neurons in the PVN are the primary target for the NT-induced actions on the HPA axis.

Until recently, the physiological relevance of the effects of NT was hampered by the lack of selective NT antagonists that could specifically block the NT effects at the level of their interaction with specific receptors. Two NT receptors have been now cloned, high-affinity levocabastine (an antihistaminergic antagonist)-insensitive and low-affinity levocabastine-sensitive receptors (Tanaka *et al.*, 1990; Chalon *et al.*, 1996).

Until now, only the high-affinity receptor has been pharmacologically investigated in term of nonpeptide NT receptor antagonists (Gully *et al.*, 1993). The first non-peptide NT receptor antagonist, SR 48692, was shown to block the binding of radiolabeled NT in brain tissues derived from several species including humans, as well as in human colon cells HT29 and COS-7 cells transfected with the high-affinity NT receptor cDNA (Gully *et al.*, 1993). Acute administration of SR 48692 has been shown to reverse several behavioral effects observed following centrally injected NT (Poncelet *et al.*, 1994; Steinberg *et al.*, 1994) and chronic administration of the antagonist to upregulate brain NT receptors (Azzi *et al.*, 1994).

Effects of Intracerebroventricular NT Receptor Antagonist Administration on Basal and NT-induced HPA Activity

When blood samples were taken from freely-moving control rats bearing a permanent polyethylene cannula guided into the right lateral cerebral ventricle, no significant effect of the antagonist was observed on basal ACTH and CORT levels. However, icv injection of 0.6 nmol NT strongly increased circulating ACTH levels by seven- and tenfold, 30 and 60 minutes postadministration, respectively (Nicot *et al.*, 1994). The same pattern was observed with plasma CORT levels, with NT inducing a three- to fourfold increase. Pretreatment of animals with 10 nmol of the NT receptor antagonist completely blocked the NT-induced HPA activation at 30 or 60 minutes after the NT injection (Nicot *et al.*, 1994; Rostène *et al.*, 1995).

Effects of Chronic NT Receptor Antagonist Implantation Nearby the PVN on Basal and NT-induced HPA Activity

In order to check the possibility that central endogenous NT can interfere with the basal or induced activity of the HPA axis, and to characterize its site of action in the hypothalamus, rats were stereotactically implanted with bilateral 22-gauge stainless steel cannulae filled with 150 mg crystalline SR 48692 or cholesterol as control, aimed to terminate 0.5 mm dorsal to the PVN. Most cannulae were placed in the dorsal margin of the PVN so that VP expressing neurons in the magnocellular part of the PVN and CRF expressing neurons in the parvocellular part of the nucleus were not damaged by the cannula implantation, as shown by *in situ* hybridization (Nicot *et al.*, 1997). Animals handled thereafter daily for 1 minute over 5 days in order to minimize handling stress showed no significant modifications in ACTH and CORT basal plasma levels during the AM phase between control- and SR 48692-implanted rats (Figure 8.1). However, chronic bilateral SR 48692 implantation close to the PVN resulted in a twofold increase in basal VP plasma levels (1.4 ± 0.3 vs 2.2 ± 0.3 pg/ml) while oxytocin levels were not significantly affected (Nicot *et al.*, 1997).

Plasma ACTH and CORT are known to present a circadian variation in rats, with high levels in the afternoon (PM phase) as compared to morning values (AM phase). Under such conditions, SR 48692 implants were able to significantly blunt the increase of plasma ACTH and CORT observed during the brain drive of the HPA activity in the PM phase (Figure 8.1).

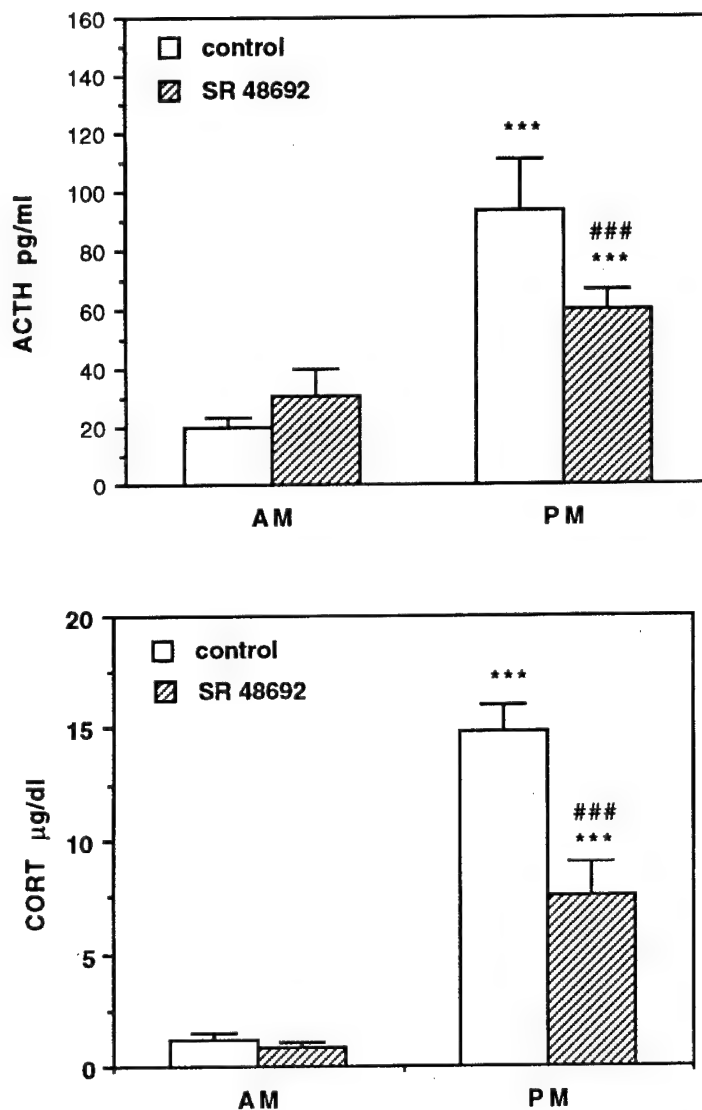


Figure 8.1 Effect of cholesterol (control) or SR 48692 implants near the hypothalamic paraventricular nucleus on the circadian rhythms of plasma ACTH and Corticosterone (CORT). *** $p < 0.01$ vs AM respective values; ### $p < 0.01$ vs control PM values. $n = 13-15$ animals/group.

Consistent with this study on circadian rhythm, exposure of the animals to a novel environment stress by placing them in a new cage resulted after 30 minutes in an increase in the HPA axis activity which was reduced in implanted SR 48692 rats (Figure 8.2).

These data suggest that NT, at the level of the PVN, controls in an opposite fashion the secretion of ACTH and VP. Together with the results of Rowe *et al.* (1997)

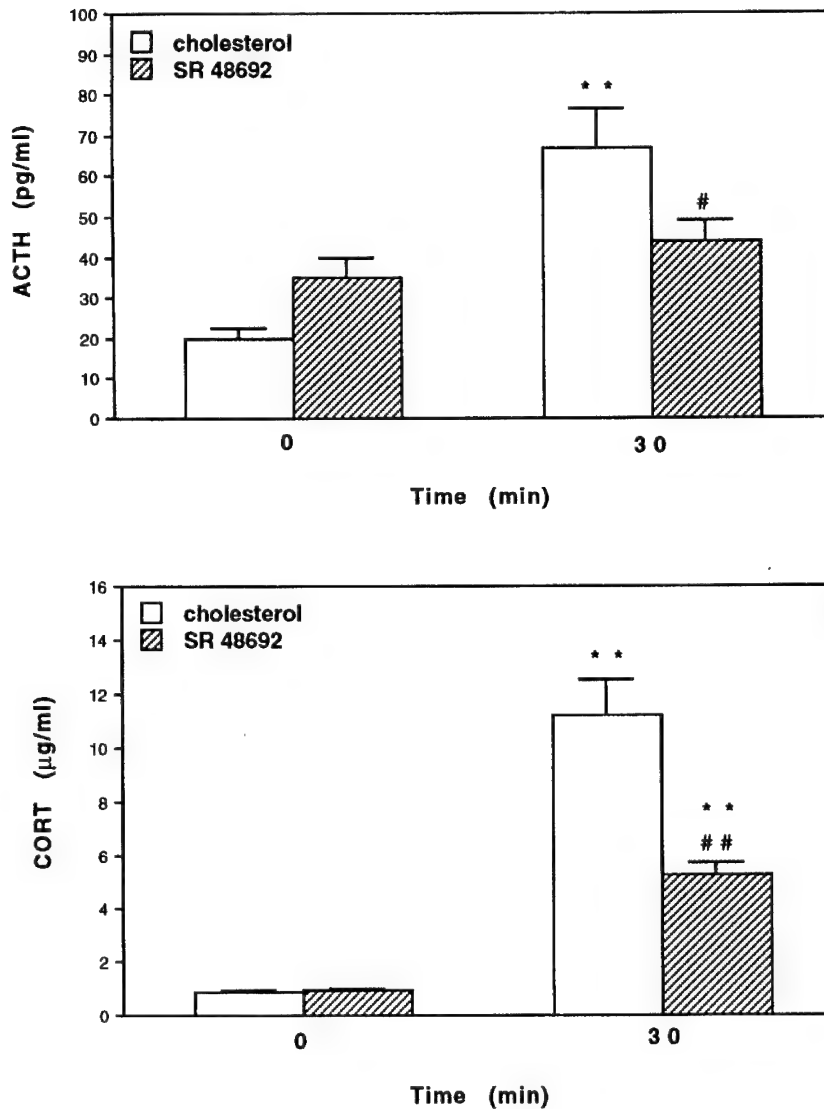


Figure 8.2 Effect of novelty stress on ACTH and CORT plasma levels at 30 minutes in rats bilaterally implanted with cholesterol (control) or SR 48692 near the hypothalamic paraventricular nucleus. ** $p < 0.01$ vs time 0; # $p < 0.05$ vs control rats; ## $p < 0.01$ vs control rats. $n = 13-15$ animals/group. Adapted from Nicot *et al.*, 1997.

showing that similar implants of SR 48692 can reduce increased responses of ACTH and CORT to restraint stress, the present findings indicate that the inhibitory effect of NT antagonists on HPA activity is restricted to a period of enhanced brain drive during which the release of CRF occurs. They also strongly suggest that the PVN represents an important target mediating the NT-induced stimulatory effect on HPA axis.

GLUCOCORTICOID EFFECTS ON CENTRAL NT SYSTEMS

Conversely to the effect of NT on CORT reported above, GCs themselves may influence the central activity of NT. Mild electrical footshock stress in the rat increased NT concentrations in the discrete dopaminergic cell body groups of the lateral ventral tegmental area (Kilts *et al.*, 1992). Moreover, ether or immobilization stress was shown to increase NT mRNA in the PVN in the rat (Watts, 1991; Ceccatelli and Orazzo, 1993). Hypercorticism induced by a chronic subcutaneous implant of CORT resulted in a selective induction of NT mRNA expression in the periventricular and rostral arcuate nuclei of the hypothalamus, as well as in the central nucleus of the amygdala (Nicot *et al.*, 1995; Watts and Sanchez-Watts, 1995). Notably, the same treatment did not alter the expression of NT mRNA appreciably in the PVN, lateral hypothalamus, or CA1-CA2 region of hippocampus (Nicot *et al.*, 1995; Watts and Sanchez-Watts, 1995). This selective effect of CORT could be involved in neuroendocrine changes observed following GCs administration. In the absence of CORT, aldosterone increased NT mRNA accumulation in the central nucleus of the amygdala (but again not in the PVN) and blunted the increase in NT expression observed in the amygdala following CORT (Watts and Sanchez-Watts, 1995). The *in vivo* functional effect of GCs in the mediobasal hypothalamus on NT expression may be direct since high GR-receptor immunoreactivity has been detected in hypothalamic NT neurons (McEwen *et al.*, 1986; Ceccatelli *et al.*, 1989). It may also be related to the observation that GCs treatment (Gudelsky *et al.*, 1989), as well as injection of NT into the cerebral ventricle (Taché *et al.*, 1979), inhibit stress-induced release of prolactin, suggesting that CORT-stimulated NT neurons of the periventricular hypothalamus may be involved in the regulation of this pituitary hormone. This action could be secondarily mediated by the tuberoinfundibular dopaminergic neurons, which exert an inhibitory control on prolactin secretion and are activated by NT (Gudelsky *et al.*, 1989).

The participation of GCs in the modulation of NT production was also investigated *in vitro* on cell lines of different origins. It was shown that dexamethasone (DEX) increased levels of NT and different NT/Neuromedin precursor-derived products as well as the amounts of the NT mRNA in cells derived from a transplantable rat medullary thyroid carcinoma (Zeytinoglu *et al.*, 1983; De Nadai *et al.*, 1993). Moreover, in pheochromocytoma PC12 cells derived from a rat adrenal medullary tumor, synergistic effects of nerve growth factor (NGF), GCs, activators of adenylate cyclase, and lithium were observed on NT production and on the amount of NT mRNA (Tischler *et al.*, 1986; Dobner *et al.*, 1988; Caillaud *et al.*, 1995). This multifactorial regulation was consistent with the presence in the NT gene of several sequences similar to the consensus cAMP response element (CRE), the glucocorticoid response element (GRE), and the AP-1, the binding sequence of the protooncogenes c-fos and c-jun (Dobner *et al.*, 1987). Nevertheless, in spite of these potentialities offered by the promoter sequence, no synergy between GCs and the aforementioned factors was evidenced in the rMTC cell lines (De Nadai *et al.*, 1993), indicating that the operant regulations were highly dependent upon the machinery of the host cell. Recent studies on primary culture of fetal hypothalamic neurons also showed that DEX enhanced NT mRNA and peptide expression, and evidenced a

synergy between GCs and the adenylate cyclase activator in this regulation (Scarcériaux *et al.*, 1995).

Hypothalamic neurons from 17-day-old rat embryo were grown for 10 days in a defined culture medium in order to obtain a maximum level of endogenous NT in the cells (Scarcériaux *et al.*, 1994). The primary cultures were then treated for 2 days with GCs and/or forskolin, an activator of adenylate cyclase activity. Under these conditions, DEX induced a dose-dependent increase in intracellular NT levels, with a maximal effect occurring at $0.1 \mu\text{M}$ representing a 100% increase (Scarcériaux *et al.*, 1995). In order to test the selectivity of the DEX effect, different glucocorticoid agonists or antagonists were used. This increase of DEX on NT was mimicked by the glucocorticoid agonist, RU 28362, and blocked by the glucocorticoid antagonist, RU 38486, suggesting that the DEX action was glucocorticoid-receptor mediated (Scarcériaux *et al.*, 1995; Rostène *et al.*, 1995). The increase in NT content induced by DEX was also associated with several changes of the NT system: (1) DEX treatment enhanced the number of hypothalamic immunoreactive NTergic cells; (2) the increase in NT immunoreactivity due to DEX was associated with an increase in NT mRNA as shown by Northern blot analysis; and (3) DEX produced an increased accumulation of NT in the medium (Figure 8.3).

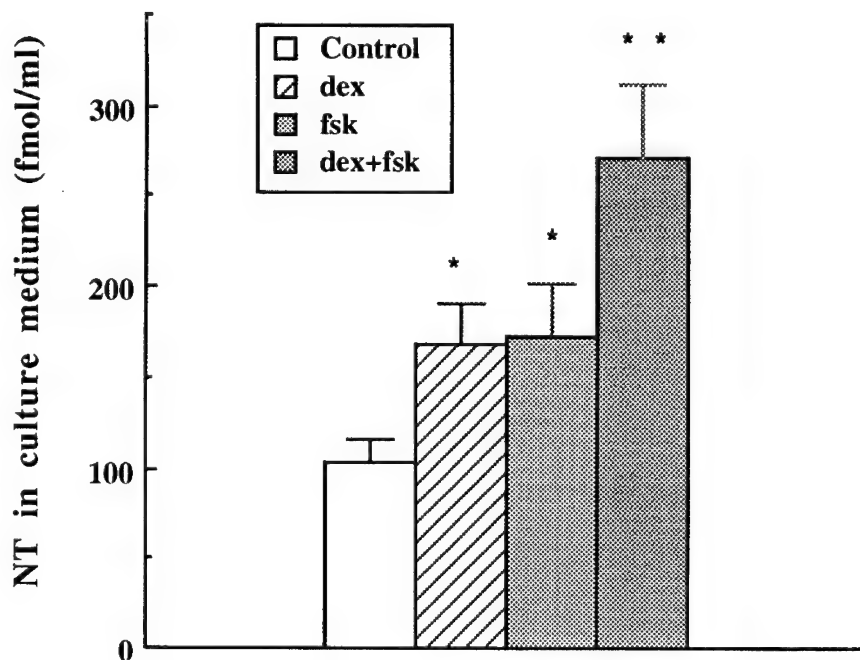


Figure 8.3 Effect of dexamethasone (dex) and/or forskolin (fsk) on the accumulation of NT in the culture medium of hypothalamic cells. Hypothalamic cells were treated from day 10 of culture for 48 hours by dex ($1 \mu\text{M}$), fsk ($1 \mu\text{M}$) or both, and the amount of NT present in the medium was measured by radioimmunoassay. Each value represents the mean \pm SEM of 3 culture wells and statistical differences versus control were assessed by ANOVA followed by Newman-Keuls test (* $p < 0.05$; ** $p < 0.01$). Adapted from Scarcériaux *et al.*, 1996.

These findings suggested that DEX increased the synthesis and the levels of NT, inducing a subsequent enhanced release of the peptide by hypothalamic neurons. When incubated with the activator of adenylate cyclase forskolin, the effects of DEX were highly potentiated (Scarcériaux *et al.*, 1995; Scarcériaux *et al.*, 1996).

Furthermore, whereas DEX alone did not affect the binding properties of ^{125}I -NT on these hypothalamic cells, the combined treatment with forskolin induced a 40% decrease in the number of NT binding sites (1.56 ± 0.12 fmol/well in control cultures vs 1.11 ± 1.11 fmol/well in treated cells) without any modification of the dissociation constant value (0.55 ± 0.03 nM vs 0.61 ± 0.03 nM) (Scarcériaux *et al.*, 1996). These data have been confirmed by the quantitation of NT receptor mRNA by RT-PCR showing that the treatment by DEX plus forskolin was associated with a 68% decrease in the number of molecules of NT receptor mRNA per μg total RNA (Figure 8.4).

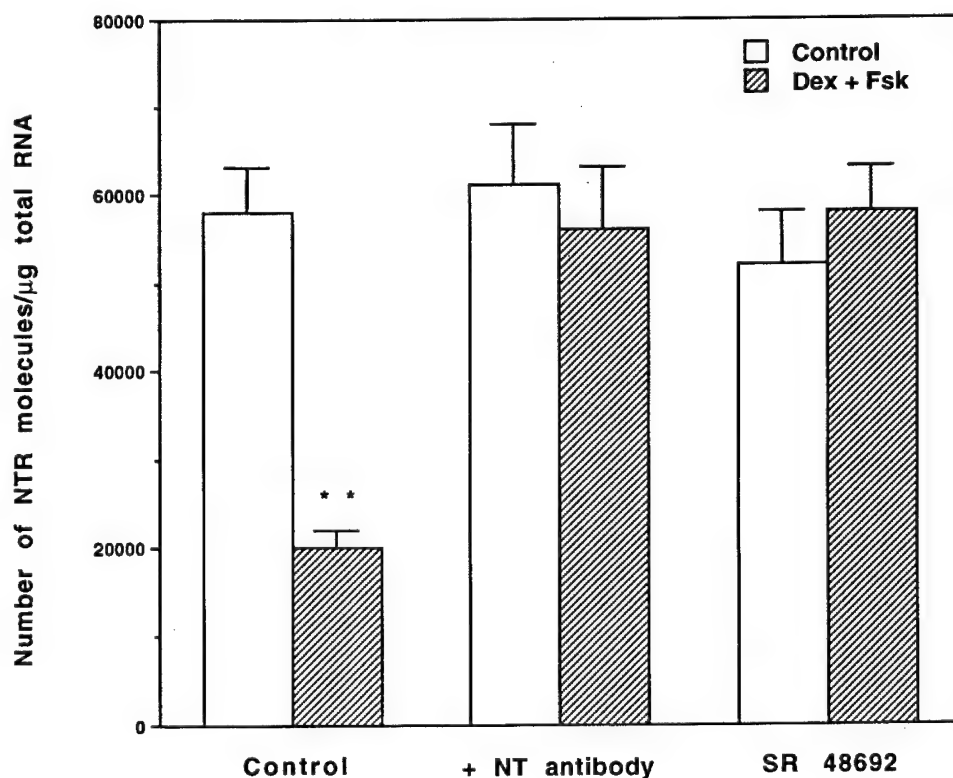


Figure 8.4 Effect of anti-NT immunoglobulins (IgG) and SR 48692 on the decrease in Neurotensin receptor (NTR) mRNA induced by the dexamethasone (Dex) plus forskolin (Fsk) treatment. Hypothalamic cells were treated from day 10 of culture for 48 hours with $1\mu\text{M}$ Dex and $1\mu\text{M}$ Fsk, with or without $1/100$ IgG or $1\mu\text{M}$ SR 48692. Each value represents the mean \pm SEM of 3 cultures and statistical differences versus the corresponding control were assessed by ANOVA followed by Newman-Keuls test (** $p < 0.01$). Adapted from Scarcériaux *et al.*, 1996.

What can be the mechanism by which DEX and forskolin affect the NT receptor capacities? A possibility is a direct effect on the binding of NT. However, as seen above, DEX alone was unable to affect the binding properties of ^{125}I -NT. It is known that NT receptors, like other peptide or monoaminergic receptors, can be downregulated by their own ligand. A decrease in the number of cell-surface NT binding sites after exposure to agonist has been previously described in several cell lines (Chabry *et al.*, 1995) and in primary cultures of forebrain neurons (Vanisberg *et al.*, 1991). This decrease was associated with an agonist-induced internalization of the ligand-receptor complex (Vanisberg *et al.*, 1991). Since DEX and forskolin can enhance the release of NT, it was thus possible that the decrease in NT binding could be due to the increased concentration of the peptide near its binding sites. Indeed, we recently supported this hypothesis showing that a nondegradable agonist of NT, JMV449, (Dubuc *et al.*, 1992) was able to reproduce the decrease in the expression of NT receptor mRNA molecules as observed following DEX and forskolin (Scarcériaux *et al.*, 1996). Furthermore, coincubation of DEX and forskolin with either an antibody against NT or the nonpeptide receptor antagonist, SR 48692, was able to counteract the inhibitory effect of DEX and forskolin on NT receptor (Figure 8.4).

All these data indicate that GCs and activators of adenylate cyclase can indirectly downregulate NTR synthesis by increasing NT release. The present findings on NT-induced downregulation of the NT receptor synthesis are in agreement with a recent *in vivo* study, in which we showed that a chronic treatment with SR 48692 led to increased levels of NT receptor mRNA in the ventral mesencephalon (Azzi *et al.*, 1994), suggesting that the NT receptor synthesis is similarly under an inhibitory action of NT *in vivo* (Figure 8.5).

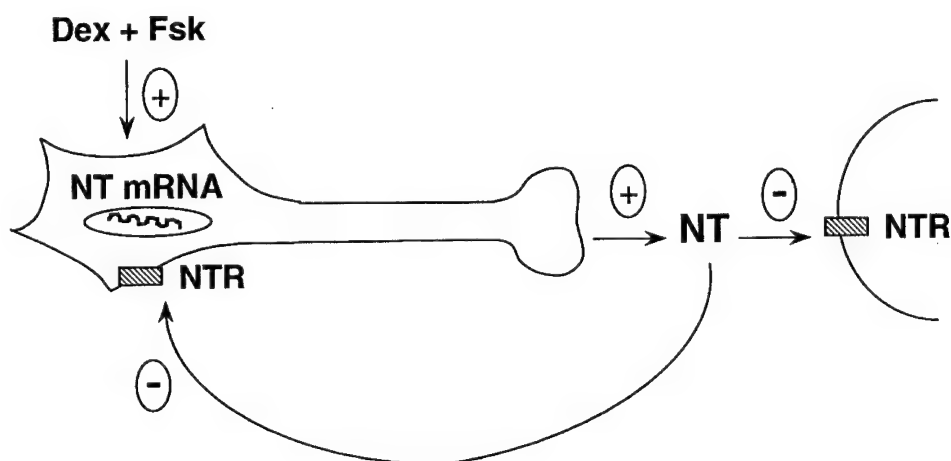


Figure 8.5 Schematic drawing representing the possible effects of dexamethasone (Dex) and forskolin (Fsk) on hypothalamic NT. This treatment increases NTmRNA expression, intracellular NT levels and NT release. The enhanced concentration of the peptide in the synaptic cleft can down-regulate NT receptors (NTR) located either postsynaptically or presynaptically as autoreceptors on hypothalamic neurons.

CONCLUSION

Previous studies on hypothalamic cultures demonstrated that GCs decreased CRF release (Hu *et al.*, 1992), consistent with the negative feedback exerted by GCs on the HPA axis activity (Kovacs *et al.*, 1986; Sawchenko, 1987). It appears, however, that hypothalamic NT levels are not under a negative feedback regulation by GCs since, as mentioned above, the latter induced an upregulation of NT production in hypothalamic neurons both *in vivo* and *in vitro*. Why GCs induced an increase in hypothalamic NT, activating in the PVN CRF neurons, and by consequence the HPA axis, remains to be elucidated. This illustrates the complexity and the time-dependency of the different peptidergic systems implicated, together with classical neurotransmitters, in the activation and inhibition of the HPA axis.

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II. Stress-Hormone Action: Basic Mechanisms

9 Corticosteroids and Calcium Homeostasis: Implication for Neuroprotection and Neurodegeneration

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The concentration of intracellular free calcium (Ca) ions, which is an important regulatory component for neuronal excitability and viability, is determined by many factors, including Ca influx, Ca-buffering, intracellular Ca sequestration, and Ca-extrusion mechanisms. Some of these factors were recently shown to be affected by corticosteroid hormones acting via intracellular receptors in hippocampal cells. At present the data indicate that predominant occupation of mineralocorticoid receptors results in a steady transmission of excitatory signals associated with low Ca influx. Concomitant activation of glucocorticoid receptors evokes a large Ca influx, resulting in a relatively strong accommodation of firing frequency. The latter, in combination with a depressed glutamate-mediated synaptic transmission, can curtail the potential overexposure to Ca ions during glucocorticoid receptor activation. However, when activation of glucocorticoid receptors is chronic or when it is linked to locally diminished inhibition or enhanced excitation, corticosteroids represent an added risk factor to neurodegenerative processes.

CALCIUM INFLUX

Calcium ions play an important role in neuronal excitability and viability (Ghosh and Greenberg, 1995). Ca influx takes place through voltage- and ligand-gated ion channels (Bertolini and Llinas, 1992; see Figure 9.1). Several subtypes of voltage-gated Ca channels have been described in the brain. These subtypes were distinguished on the basis of differences in voltage sensitivity, pharmacological profile, and, more recently, in primary structure (Carbone and Swandulla, 1989; Varadi *et al.*, 1995). It was shown that Ca channels are multisubunit complexes of which the $\alpha 1$ -subunit forms the ion pore and provides for voltage-dependent gating (Dunlap *et al.*, 1995). Using radioactively labeled proteins binding selectively to the channels or *in situ* hybridization for $\alpha 1$ -subunit mRNAs, specific regional distribution patterns were observed (Tanaka *et al.*, 1995).

Hippocampal neurons carry a variety of Ca-channel subtypes. L-type channels, in part formed by $\alpha 1C$ and/or $\alpha 1D$ subunits, are located on proximal dendrites of pyramidal neurons (Hell *et al.*, 1993). The conductance of the channels is ~ 25 pS

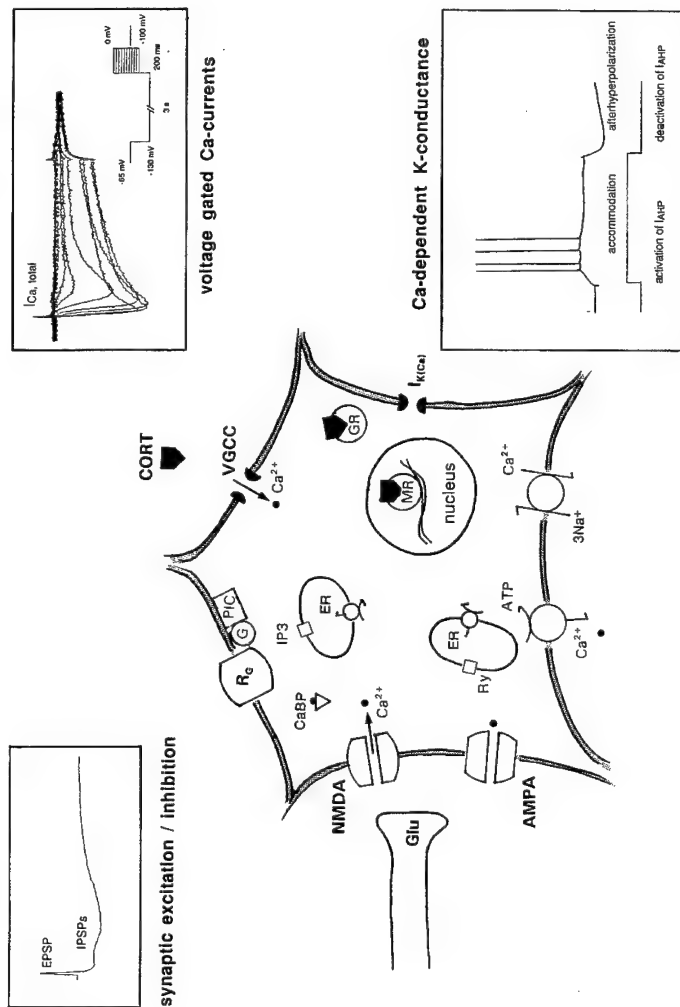


Figure 9.1 Schematic overview of cellular processes important for the intracellular Ca-concentration. Ca influx takes place through voltage gated Ca-channels (VGCC) and ligand gated Ca-channels, such as the NMDA and (to a lesser extent) the AMPA receptors. Typical examples of the electrical signals caused by these two routes of Ca influx are shown on the upper right and left, respectively. Ca-ions can also be released from intracellular stores, e.g. through increases of IP_3 caused by activation of a G-protein coupled receptor (R_G) or through activation of ryanodine (Ry) receptors on the endoplasmic reticulum (ER). Ca-binding proteins contribute to the buffer capacity of the cell: Free Ca-ions amount to only 1/100 of the total intracellular $[Ca]_i$. Extrusion mechanisms such as the Ca-ATPase and the Na/Ca antiporter enable the cell to transfer excess Ca-ions to the extracellular compartment. Temporary changes in the intracellular Ca concentration can be directly calculated from single cell Ca-imaging experiments (see Figure 9.2). However, indirect information can also be obtained by recording Ca-dependent conductances, such as the slow Ca-dependent K-conductance which causes firing frequency accommodation during a depolarizing pulse as shown on the lower right. The cellular processes linked to Ca-homeostasis are a potential target for corticosteroids acting through intracellular mineralocorticoid (MR) and glucocorticoid receptors (GR).

and gives rise to long-lasting currents, with little voltage-dependent inactivation. The activation of the channels does show voltage dependency, in that these channels only open upon strong depolarization (high threshold activation). This indicates that L-type Ca currents are prominent during sustained, large depolarizations. L-type Ca currents are selectively blocked by dihydropyridines (e.g., nimodipine).

N-type Ca channels are located presynaptically, where they may be involved in neurotransmitter release, and postsynaptically, on dendrites of pyramidal neurons (Wheeler *et al.*, 1994). These channels contain an $\alpha 1B$ subunit and have a lower conductance (12–18 pS). Activation of the N-type channels evokes a slowly decaying current with a high threshold for activation; these channels also display voltage-dependent inactivation. This means that N-type channels are primarily active shortly after a neuron at resting membrane potential (RMP) receives a strong depolarizing input. N-type Ca channels are selectively blocked by ω -conotoxin GVIA. Similar high threshold, slowly decaying currents are generated by P-type channels which were also demonstrated in the hippocampal region (Wheeler *et al.*, 1994). P-channels contain an $\alpha 1A$ subunit. The P-type currents are most efficiently distinguished from N-type currents by their sensitivity to the blocker ω -Aga IVA (Llinas *et al.*, 1992).

The last of the presently well characterized hippocampal Ca currents is the transient (low threshold) T-type current. This current can be activated at approximately RMP, but shows strong voltage-dependent inactivation. Therefore, it is likely to contribute only transiently to Ca influx, particularly when neurons display rather negative membrane potentials. Specific blockers for the T-type currents are presently unavailable. While a subunit ($\alpha 1E$) giving rise to low-threshold, transient currents in expression systems has been identified (Dunlap *et al.*, 1995), it is presently unclear whether the T-type Ca current in hippocampal cells is generated by channels containing this subunit.

Ca influx can also take place through ligand-gated channels. Two groups of channels (i.e., AMPA and NMDA receptors) were found to be particularly important. These receptors, activated by the neurotransmitter glutamate, also consist of an assembly of subunits (Seeburg, 1994). NMDA receptor channels conduct Na, K and Ca ions. The duration of the response, and thus the extent of Ca influx, depends on the ratio in which the various subunits are present (Monyer *et al.*, 1992). Ca influx through AMPA receptors is usually very limited. The Ca conductance of AMPA receptors becomes noticeable only when the GluR2 subunit is reduced in number relatively to other subunits (Hollmann *et al.*, 1991).

CALCIUM BUFFERING AND EXTRUSION

The regulation of cellular processes by Ca depends on the intracellular concentration of free Ca ions ($[Ca]_i$) at a given point in time, but also on the duration of the changes in $[Ca]_i$. Changes in $[Ca]_i$ are partly determined by the influx of Ca ions from extracellular sources. However, Ca release from intracellular stores, such as Ca-induced Ca release via ryanodine receptors and Ca release via IP3 receptors are also important (Simpson *et al.*, 1995).

Transient elevations of $[Ca]_i$ are controlled by several mechanisms (Clapham, 1995). One is linked to the presence of Ca-binding proteins. Of these, calmodulin, calretinin, calbindin and parvalbumin have been demonstrated in various subgroups of hippocampal cells. A second mechanism is provided by sequestration into intracellular compartments (e.g., via endoplasmatic reticulum Ca-ATPases). Ultimately, Ca ions must be transported back to the extracellular space via Ca-ATPases or the Na-Ca antiporter.

Ca influx and its functional consequences can be studied by a number of recently developed techniques (Figure 9.1). Thus, Ca influx via voltage-gated channels can be recorded with microelectrodes or patch electrodes, under voltage clamp conditions. The dynamic range and temporal aspects of changes in intracellular Ca level $[Ca]_i$ can be monitored by single-cell imaging methods, using fluorescent Ca-sensitive probes such as fura-2. Finally, the potential functional consequences of changes in Ca levels can be examined (e.g., by recording phenomena linked to activation of the slow Ca-dependent K conductance). Activation of this conductance underlies the attenuation of cell firing during a steady depolarization (accommodation) and the subsequent afterhyperpolarization (AHP) of the membrane.

CORTICOSTEROIDS AND Ca HOMEOSTASIS

Calcium Influx

Ca influx and homeostatic control of intracellular Ca levels in hippocampal cells are a potential target for steroid hormones acting via mineralo- and glucocorticoid receptors (MRs and GRs respectively, see de Kloet, this volume). Recent electrophysiological, Ca-imaging and molecular studies have shown that steroids can indeed control these features over a prolonged period of time.

In voltage clamp studies it was found that a predominant activation of MRs in CA1 pyramidal neurons, as occurs during rest at the circadian trough, results in small low- and high-threshold Ca currents (Karst *et al.*, 1994). When tissue was exposed to higher doses of corticosterone, resulting primarily in activation of the remainder of the GRs, an increase in particularly high-threshold Ca currents was observed (Kerr *et al.*, 1992; Karst *et al.*, 1994). When neither MRs nor GRs were occupied (i.e., in tissue from adrenalectomized [ADX] rats), Ca current amplitude was increased compared to sham-operated, mildly stressed control animals (Karst *et al.*, 1994). This points to a U-shaped dose dependency of modulatory effects by steroids on Ca conductance and on Ca influx. The extent of the change evoked by ADX and the nature of the Ca conductance most sensitive to ADX appeared to depend on the developmental stage of the animal. At a young age (1 month) ADX mainly increased the amplitude of the high-threshold Ca current, while at a later age (6 months) the effect was most apparent on low-threshold Ca currents.

There are indications that a certain degree of interaction between functional MRs and GRs is necessary to accomplish this modulation of Ca influx. In an animal model where GRs are completely absent (homozygous GR-knockout mouse), Ca

currents were large in amplitude and indistinguishable from currents observed in ADX mice (Hesen *et al.*, 1996). This points to the necessity of functional GRs to accomplish MR-mediated events. The results obtained with a selective GR-agonist in tissue from ADX rats have been somewhat conflicting and may depend on agonist dose (Kerr *et al.*, 1992; Karst *et al.*, 1994).

The effect of corticosteroids on Ca influx through ligand-gated Ca channels has not been specifically investigated. Indirect evidence for such a role can be inferred from experiments showing that primed burst potentiation in the hippocampus, a phenomenon closely linked to NMDA receptors (see Diamond, this volume), depends on relative MR and GR occupation. Predominant occupation of MRs is associated with potent primed burst potentiation in both dentate gyrus and in CA1. Additional full activation of GRs depressed primed burst potentiation, in some cases even revealing long term depression of amino-acid-mediated synaptic input. In tissue from ADX rats, primed burst potentiation was also limited, pointing to an inverted U-shaped dose-dependency.

Calcium Buffering and Extrusion

In hippocampal cultures, basal $[Ca]_i$ appeared to be elevated after prolonged exposure to very high corticosteroid levels, probably acting via GRs (Elliott and Sapolsky, 1993). When these cultures were depolarized by kainic acid, increases in $[Ca]_i$ were more marked after exposure to high corticosteroid doses. This study suggested that the intracellular Ca buffering and the overall change in $[Ca]_i$ after depolarization are affected by corticosteroid hormones, although the specific effects of MR and GR activation were not revealed.

We recently studied this issue using a combined electrophysiological and single-cell Ca-imaging approach in acutely dissociated hippocampal cells that were treated with selective MR and GR analogues prior to dissociation. The patch pipette which was used for recording of voltage-dependent Ca currents under whole-cell voltage-clamp conditions contained 100 μM of the calcium indicator fura-2; approximately 5 min were allowed for diffusion of the dye into the cell processes (see legend of Figure 9.2 for more experimental details). The cell was subjected to a sequence of seven depolarizing voltage steps, thus allowing Ca influx through low- and high-threshold voltage-gated Ca channels. During and after these pulses, the $[Ca]_i$ was calculated: changes in $[Ca]_i$ result from an altered Ca influx, which was recorded with electrophysiological means, and unknown processes such as Ca-dependent release from intracellular stores, buffering and Ca-extrusion mechanisms. Steroid sensitivity appeared to be particularly clear for the recovery of the Ca signal back to baseline level. When the Ca signals in the different treatment groups were normalized to the maximal value at the end of the depolarizing pulses, cells in which both MRs and GRs were activated 1–4 hr before the experiment displayed a significantly slower recovery than neurons displaying predominant MR occupation. These preliminary findings indicate that GR activation (in addition to MR activation) not only results in a larger Ca influx, but that the elevation of $[Ca]_i$ under these circumstances is also much more prolonged.

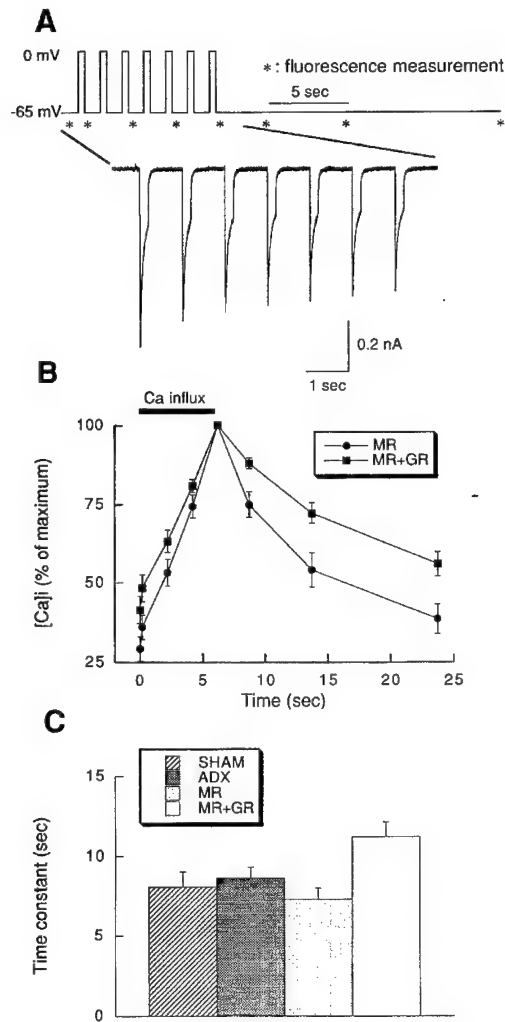


Figure 9.2 Recovery of intracellular calcium levels in acutely dissociated CA1 hippocampal neurons following calcium influx through voltage-dependent calcium channels. (A) Example of calcium currents (lower traces) evoked by seven consecutive 200-msec depolarizing voltage steps to 0 mV (upper traces; holding potential: -65 mV). Calcium currents were recorded, using the whole-cell voltage clamp technique, with electrodes containing the calcium indicator fura-2 ($100 \mu\text{M}$). The asterisks indicate at which time points fluorescence measurements, with the digital ratio technique, were made to monitor intracellular calcium levels. (B) Normalized calcium influx and recovery of the calcium signal (calcium levels after seventh voltage step as the 100% level) in CA1 pyramidal neurons. Acutely dissociated neurons were obtained from slices of adrenalectomized rats that were treated with 30 nM corticosterone (MR + GR) or 30 nM corticosterone and 500 nM of the GR antagonist RU 38486 (MR). A time constant for the recovery was obtained by fitting the decaying phase with a single exponential function. (C) Time constants for the recovery of the calcium signal following calcium influx. Acutely dissociated CA1 neurons were obtained from slices of sham operated or adrenalectomized (ADX) animals. Control ADX slices were not treated with steroids (MR and GR unoccupied) whereas MR and MR + GR were selectively occupied as described above. Neurons with predominant MR occupation have a significantly lower time constant for the recovery of the Ca-signal than neurons where both MRs and GRs were occupied.

Calcium Dependent Potassium Conductance

Corticosteroid dependent modulation of Ca influx and buffering in hippocampal cells will have implications for cellular phenomena which depend on $[Ca]_i$. The best studied example is the slow Ca-dependent K conductance. With predominant MR occupation the accommodation and AHP were found to be comparatively small (Joëls and de Kloet, 1990). From this one can conclude that, when corticosteroid levels are low, excitatory input is efficiently transferred. Additional GR activation resulted in an enhanced accommodation and AHP amplitude, so that excitatory input is attenuated (Joëls and de Kloet, 1989; Kerr *et al.*, 1989).

In the absence of corticosteroids, when MRs and GRs are unoccupied, the accommodation and AHP amplitude were at an intermediate level (Joëls and de Kloet, 1989; Kerr *et al.*, 1989). This illustrates that Ca influx is not the only determining factor for the accommodation and AHP amplitude: based on Ca currents one would expect to see a much more pronounced accommodation and AHP amplitude. Additional effects of corticosteroids on either Ca-extrusion, -buffering, -diffusion, or even on the Ca-dependent K channel itself can explain this discrepancy.

IMPLICATIONS FOR NEUROPROTECTION AND NEURODEGENERATION

The experimental data show that corticosteroid hormones modulate Ca homeostasis in one of the target areas for the hormone, the hippocampus. The steroid effects are slow in onset and of prolonged duration. The steroid modulation of Ca currents and Ca-dependent phenomena was not seen in the presence of a protein synthesis inhibitor, pointing to the involvement of nuclear receptors (Karst and Joëls, 1991; Kerr *et al.*, 1992). The actions described herein are not likely to be caused by metabolites of corticosteroid hormones acting via membrane recognition sites. While effects of A-ring reduced steroids on Ca currents have been reported, those actions were rapid in onset and readily reversible (French-Mullen *et al.*, 1994).

At this moment, the modulatory role of corticosteroids on intracellular Ca levels is only partly understood. This particularly pertains to the dynamics and temporal aspects of fluctuating Ca levels, which are important to appreciate the impact of steroid mediated effects for Ca-dependent phenomena. The presently available data indicate that conditions of predominant MR activation result in small Ca influx and relatively rapid recovery of the intracellular Ca signal. When GRs are additionally activated, Ca influx is increased; temporary rises in $[Ca]_i$ are slowly restored, so that cellular phenomena that depend on the overall Ca level (e.g., I_{AHP}) are large. When steroids are absent, a more complicated situation arises. Electrophysiological and imaging data indicate that local Ca influx, particularly in the dendrites, is large; however, the accommodation and AHP were found to be rather small, suggesting that efficient buffering and/or extrusion mechanisms prevent prolonged rises in $[Ca]_i$.

These steroid-induced effects on Ca homeostasis should be considered in the context of other properties of hippocampal networks, such as the local balance between excitatory and inhibitory input: most of the Ca entry through voltage-gated

Ca channels will take place when the membrane is strongly depolarized. This can occur when the excitatory input is strengthened or the inhibitory input is attenuated. There are presently no indications that the balance between excitatory and inhibitory input is directly affected by corticosteroid hormones, over the same time range as is the Ca homeostasis. Thus, it was observed that MR activation is associated with a stable or even enhanced glutamate-mediated transmission (Joëls and de Kloet, 1993). Additional GR activation reduced both excitatory and inhibitory amino acid mediated input. However, these effects were relatively rapid in onset (approximately 20 min) and reversible within 2 hr.

The implications of these steroid actions on Ca homeostasis for cellular excitability during fluctuations in circulating corticosteroid levels are considerable. With predominant MR activation, such as occurs under rest at the circadian trough, accommodation and AHP amplitude are small; steady excitatory input will be effectively transmitted. The excitation is, however, associated with a limited Ca influx and rapid recovery of Ca signal, so that the overall rise of $[Ca]_i$ is restricted. When GRs are additionally activated (e.g., after an acute stress), voltage-dependent Ca influx may become much larger. Yet, the combination of a decreased response to glutamate-mediated input and a strong accommodation will curtail the potential overexposure to Ca ions. If, through other causes, the excitation is increased or inhibition decreased, one can predict that these curtailing mechanisms are no longer sufficient to prevent exposure to large amounts of free intracellular Ca ions. This may explain why corticosteroids were reported to exacerbate the damaging effects of ischemic insults in CA1 pyramidal cells (Sapolsky and Pulsinelli, 1985).

Few experiments have addressed the question of what the implications of steroid effects on Ca homeostasis could be when corticosteroid levels are chronically changed. Both chronic hyper- and hypocorticism have been described in association with a number of stress-related disorders. A limited number of studies suggest that these conditions could lead to enhanced Ca influx in CA1 hippocampal cells. First, heterozygous GR knockout mice, which display hypercorticism probably due to a reduced number of functional GRs at feedback sites, have increased high-threshold Ca currents (Hesen *et al.*, 1996). Second, a single-cell mRNA amplification study has shown that chronic treatment of ADX rats with high corticosteroid levels in the drinking water leads to an increased mRNA expression for $\alpha 1A$ and $\alpha 1C/D$ Ca channel subunits (Werkman *et al.*, 1996); the proteins encoded by these subunits are responsible for sustained high-threshold Ca currents. Chronic treatment with low corticosteroid levels resulted in the opposite effect (i.e., very low mRNA expression levels for $\alpha 1A$ and $\alpha 1C/D$ subunits). In this study, chronic ADX (without steroid replacement) evoked an increase in the $\alpha 1C/D$ subunit mRNA level. This agrees with earlier findings that animals which were at least 3–4 days ADX showed large Ca current amplitudes.

Prolonged exposure to a high $[Ca]_i$ introduces a potential threat to neuronal viability (Choi, 1988). We therefore hypothesize that chronic hypo- or hypercorticism, through changes in Ca homeostasis, could lead to neurodegenerative processes. This may contribute to the atrophy of distal dendrites of pyramidal neurons observed with chronic exposure to very high corticosteroid levels (Woolley *et al.*, 1990; Levy, this volume) and to apoptosis of dentate granule cells taking place

several days after ADX (Sloviter *et al.*, 1989). Conversely, predominant MR activation may exert neuroprotective effects in the hippocampus. Clearly, a combined electrophysiological, Ca-imaging and morphometric analysis of these phenomena at timed intervals after introducing the aberrant steroid level is required to support this hypothesis.

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10 Deleterious Consequences of Corticosteroid Exposure: Possible Therapeutic Neuroprotection

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Hippocampal neurons are extremely sensitive to corticosteroids and are endangered both by their absence and by their high concentrations. Prolonged exposure to high levels of corticosterone (CORT) was shown to induce morphological changes in the hippocampal area of rats' brains, similar to those found following chronic stress and resembling changes found in aging rats. In order to further investigate these phenomena, we implanted sustained-release (SR) CORT pellets in young and in middle-aged rats (the later previously divided to cognitively "impaired" and "nonimpaired" groups). Cognitive impairments and morphological brain changes following treatment were evaluated and correlated. Morphological changes were found mainly in CA1 and CA4 hippocampal regions, but also in CA3 and dentate gyrus (DG). Middle-aged rats were more susceptible to treatment than young ones. Fischer-344 rats seemed more sensitive than other strains tested in different studies. Extended cognitive impairment was detected only in "nonimpaired" adult rats, since a "ceiling damaging effect" was found in previously "impaired" rats. High correlation was observed between cognitive impairment and percentage of damaged cells in hippocampal regions CA3 and CA1. Prolonged CORT exposure also induced hippocampal cholinergic hypofunction in middle-aged rats three months following treatment, as measured with the microdialysis technique.

Activation of glucocorticoid (GC) receptors in CA1 neurons was reported to increase conductance of voltage dependent calcium (Ca) channels, leading to a rise in intracellular Ca and, through a cascade of events, to cell death. A concomitant treatment with the L-type Ca-channel blocker, nimodipine, blocked in young rats the morphological damage of prolonged CORT treatment. The light-microscopy findings were recently replicated in an electron-microscopy (EM) study. These data might have important implications for aging research.

Aging is accompanied in many cases by a decline in cognitive function, which is highly correlated with morphological changes in the hippocampal formation (Kadar *et al.*, 1990, 1994). However, the individual differences within any given population are striking and intriguing, raising the question whether environmental factors might contribute, in addition to predominating genetic factors, to "successful aging." With the rapid increase of life expectancy in developed countries, this might become a significant as well as fascinating subject.

Coping with stress, mainly the return to homeostasis following stressful perturbation, is also impaired with age (for an extensive review see Sapolsky, 1992). Since

the hippocampus is also involved in the feedback regulation of the response to stress (Jacobson and Sapolsky, 1991), it is reasonable that the above phenomena are somehow related. Indeed, a positive correlation between extent of morphological damage in the hippocampus and adrenal activity of rats during aging was reported by Landfield and coworkers almost two decades ago (Landfield *et al.*, 1978). The hippocampal formation, an area particularly rich in corticosteroid receptors, seemed to be especially sensitive to drastic changes in their concentration (McEwen *et al.*, 1986). A vast amount of related data that accumulated over the years led to the development of the glucocorticoid (GC) hypothesis of brain aging and degeneration (Landfield and Eldridge, 1991; Landfield, 1994), to the GC cascade hypothesis (Sapolsky *et al.*, 1986), and to additional intriguing ideas about stress-aging association (McEwen, 1992).

One unique feature of hippocampal-CORT interactions is the fact that neuronal damage also takes place under zero CORT levels following adrenalectomy, mainly at the DG region (Sloviter *et al.*, 1989; Gould *et al.*, 1990). A certain concentration of CORT might therefore be required for neuronal protection. The fact that CORT activates at different concentrations the high affinity (type I; MRs) and the low affinity (type II; GRs) receptors, may explain the different responses to various concentrations (Reul and De Kloet, 1985; De Kloet, 1992).

Stress-aging interactions, with specific emphasis on hippocampal morphological changes and their cognitive consequences, became the focus of our research. A principal pharmacological goal in this research was the attempt to find therapeutic means of preventing CORT-induced brain changes, a process which was termed "an accelerated brain aging." Since the role of the cholinergic hippocampal system both in cognitive processes and in aging is fairly established, we also included in our studies some measures of central cholinergic function. The first necessary task was to establish an animal model that would allow these studies; we chose Fischer-344 rats of different age groups, in which sustained-release (SR) CORT pellets were implanted.

THE ANIMAL MODEL — FEATURES, PROS AND CONS

Fischer-344 inbred rats have been used in many aging studies, possibly because their weight does not increase with age as much as in other strains. They were selected for the present studies in order to compare the "accelerated" aging effect of steroid treatment, especially on cognitive performance, to previously available data of normal aging. However, this strain was found to be more sensitive to stress (Dhabhar *et al.*, 1995), a fact that was recently further confirmed (Ingram *et al.*, 1996). Indeed, the use of other rat strains in similar models led to variable results (Bodnoff *et al.*, 1995; Luine *et al.*, 1993), which implies that the experimental parameters (e.g., CORT levels, treatment duration, pre-screening of rats) should be determined for each rat strain separately.

Rather than stressing the rats daily, we preferred the continuous SR administration of CORT, for the following reasons:

- (1) Stress activates various hormonal systems, while our purpose was to focus on the unique effects of corticosteroids.

- (2) SR pellets permit administration of CORT without fluctuations. In contrast, CORT levels may change following stressor exposure. In this respect, SR pellets are also more convenient than daily injections, which lead to variable CORT levels and involve the additional stress of the daily injection.
- (3) By enabling adjustment of CORT levels at will, SR pellets permit the study of dose-effect relationships.
- (4) Between-subject variations of responses to stress, with regard to CORT levels that ensue, is avoided.

On the other hand, the animal model is obviously not identical to a stressful situation, and should be regarded mainly as a research tool to study CORT effects on hippocampal integrity.

In order to better simulate a conceivable prolonged stressful situation, CORT blood levels were chosen at the range attributed to mild stress (15–25 µg/dl), which in rats approximates the physiological concentration of nocturnal peak levels. During long treatment periods with SR pellets, plasma concentrations somewhat decreased gradually (e.g., from 34 to 16 µg/dl over three months; Dachir *et al.*, 1997). The constant supply of CORT also suppressed its circadian variation (Dachir *et al.*, 1993), probably due to a negative feedback inhibition of ACTH secretion. The minimal treatment duration for any measurable effect in Fischer-344 rats was nine weeks. During the first few days following implantation of the CORT pellets, rats typically lost 10–30% of their original weight (depending on the dose and the animals' age) due to an increased metabolism. Young rats later gained weight at a rate similar to the placebo group, although they did not gain the weight lost immediately after surgery (see Figure 10.1). No other gross adverse effect was monitored; however, a systematic study (e.g., of possible effects on the immune system) is yet to be performed.

EFFECTS IN MIDDLE-AGED VERSUS YOUNG RATS

The study of CORT-induced "accelerated brain aging" at different age groups introduces an obvious methodological difficulty, since at middle-age a considerable number of the rats population reveal age-related cognitive deficits. To overcome this difficulty, we screened rats for cognitive performance, based on their ability to learn the Morris water maze (MWM) (Arbel *et al.*, 1994). The two extreme groups (fast learners and slow learners) were designated as "nonimpaired" and "impaired," correspondingly. Those with average performance were not used in the study that followed, except for histological examination of their hippocampal areas, which confirmed the high correlation between cognitive scores and percent of hippocampal damaged cells (both at CA1 and CA3). Following nine weeks of CORT treatment, the rats were tested again for their ability to learn another spatial-orientation memory task — the eight-arm radial maze (RAM). Both the MWM (Brandeis *et al.*, 1989) and the RAM (Olton, 1987) were selected because numerous studies showed that successful learning of these tasks depended heavily on hippocampal integrity.

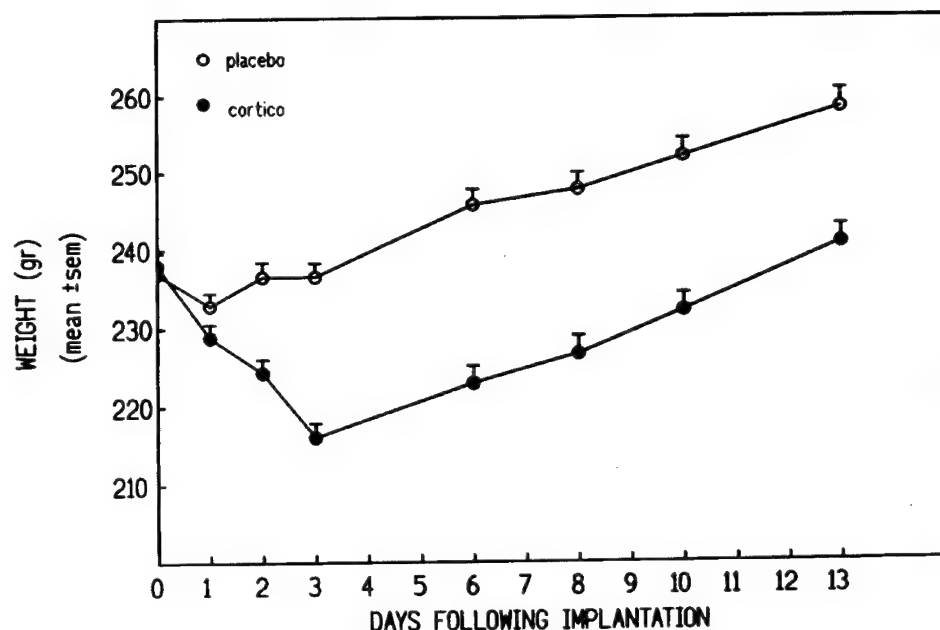


Figure 10.1 A typical example of weight changes in young rats following subcutaneous implantation of one 200mg corticosterone SR (over 21 days) or placebo pellet.

Table 10.1 Corticosterone (compared to placebo) effect on hippocampal neurotoxicity in "nonimpaired" vs. "impaired" middle-aged rats

Experimental Group	CA1 % of damaged cells	CA3 % of damaged cells
Nonimpaired/placebo	9±6	5±3
Nonimpaired/corticosterone	55±7	21±7
Impaired/placebo	47±15	17±2
Impaired/corticosterone	61±8	37±2

Of the four experimental groups ("impaired" and "nonimpaired," each treated with either placebo or CORT), only the "nonimpaired" placebo-treated group exhibited a satisfactory learning curve of the RAM. All other three groups were significantly impaired. Histological examination of the brains showed a strong correlation with the behavioral results (see Table 10.1).

In the "nonimpaired" group, CORT treatment increased the percentage of necrotic cells in CA1 from 9% to 55% (from 5% to 21% in CA3). This change seemed large enough to be expressed as cognitive deficits. The "impaired" group exhibited 47% of damaged cells following placebo treatment (17% in CA3). The behavioral scores of the CORT treated group (61% of damaged cells in CA1; 37% in CA3) were not statistically different than those of the placebo "impaired" group (though they differed from the placebo "nonimpaired"), probably because the change in

hippocampal damage was not sufficiently large. In comparison, a similar CORT treatment in young rats resulted in much lower, sometimes barely detectable, morphological changes. Longer (three-months) treatment of young rats, using somewhat higher CORT concentrations (Dachir *et al.*, 1997) resulted in measurable hippocampal injuries (see Table 10.2), although not high enough to be detected in the spatial behavioral tests (MWM and RAM). CA1 and CA3 basal levels of around 3% pyknotic cells were elevated after the treatment to approximately 23% damaged cells. Higher CA1 damages might be required to detect behavioral changes.

Accelerated neuronal loss was also reported previously in stressed 18-month old Fischer-344 rats, but not in younger animals (Kerr *et al.*, 1991). One possible explanation is that declining androgen levels with age increase hippocampal vulnerability. Indeed, one study reported that chronic stress resulted in hippocampal damage only in castrated rats (Mizoguchi *et al.*, 1992). A more recent study (Clark *et al.*, 1995) failed to corroborate this effect; thus, the critical balance between corticosteroids and androgens still requires further exploration. A somewhat related human study showed that saliva cortisol responses to stress were attenuated in women using oral contraceptives, and was larger in males compared to females (Kirschbaum *et al.*, 1995).

THE EFFECT IN VARIOUS HIPPOCAMPAL REGIONS

In a recent review, Robert Sapolsky (1996a) listed five possible mechanisms underlying the deleterious effects of GCs in the brain. Numerous studies cited in this review focused on the hippocampal formation, an area rich with GC receptors. Our microdialysis acetylcholine (ACh) measurements (see below) seem to indicate an additional injury to the medial septal area, in which cholinergic cell bodies that innervate the hippocampus are located. Within the hippocampus, most studies described damages to the CA3 region. In our own animal model, especially after short exposure of young rats, neuronal injuries were found predominately in CA1 (Levy *et al.*, 1994). It is possible that the predominantly observed damage depends on the time of observation following exposure to either stress or high CORT levels. CA1 was found to contain a higher abundance of type II (low affinity) receptors compared to CA3 (De Kloet *et al.*, 1990). It was reasonable to assume that the effect of excessively high CORT was mediated primarily via the low affinity receptors, since high affinity receptors are already highly occupied under basal conditions. Numerous studies which used GR- or MR-specific agonists proved that this was indeed the case. A recent study provided additional direct evidence of the role of low affinity receptors in age-related brain injury (Talmi *et al.*, 1996). Chronic treatment of mice from middle age to senescence with a specific type II antagonist (RU486) attenuated electrophysiological biomarkers of CA1 aging. If neuronal damage is indeed initiated at CA1, it can subsequently affect CA3 region by retrograde degeneration. The schematic innervation network of the hippocampus via the perforant path, shown in Figure 10.2 (Amaral *et al.*, 1990), indicates that the DG might be the last affected region. A detailed temporal study of the damage progression is required to examine this hypothesis.

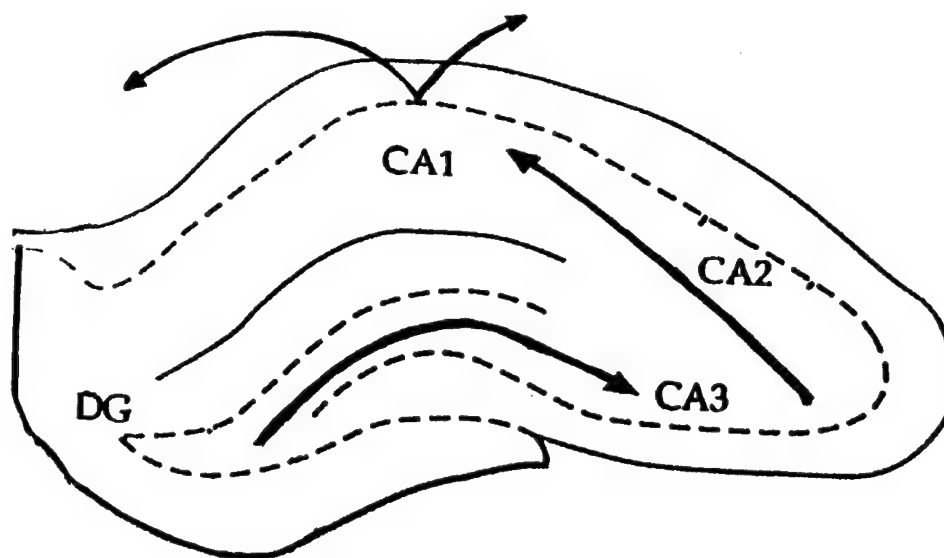


Figure 10.2 Schematic representation of the intrahippocampal innervation pathways (according to Amaral *et al.*, 1990).

CHOLINERGIC HYPOFUNCTION

The hippocampal formation plays a major part in the processing of memory and spatial-orientation processes (Jarrard, 1993), and the role of the cholinergic system in these cognitive tasks had long been established (Bartus *et al.*, 1982). With age, septal cholinergic neurons, innervating the hippocampus, were found to deteriorate, followed later by degeneration of hippocampal pyramidal cells (Gilad *et al.*, 1987; Tizabi *et al.*, 1989). Exposure of naive rats to stressful conditions, such as a new environment, induced a significant, although transient, decrease in hippocampal plasticity (Diamond *et al.*, 1990; see also the following chapter in this book).

The effects of stress and GCs on the cholinergic system is still quite controversial in the scientific literature. Some investigators reported an increase in hippocampal ACh following exposure to stress (Imperato *et al.*, 1991; Tajima *et al.*, 1996). Others found a significant decrease (Spignoli and Pepeu, 1986; Fatranska *et al.*, 1987a,b; Shukitt-Hale *et al.*, 1993; Stillman *et al.*, in press). Hypoactivity of the cholinergic system, related to memory dysfunction, was reported following prolonged stress (Zerbib and Laborit, 1990). No information exists regarding the effect of prolonged high CORT exposure on cholinergic function. In order to obtain such data, we have used the microdialysis technique, monitoring extracellular ACh levels. However, hippocampal ACh concentrations are quite low and the sensitivity of the available analytical methods (primarily HPLC) hinders detection of small concentration changes. To overcome this problem, we used scopolamine challenge before ACh measurement. Scopolamine, a nonspecific muscarinic antagonist, blocks both the post-synaptic M1 receptors and the pre-synaptic M2 autoreceptors. A blockade of

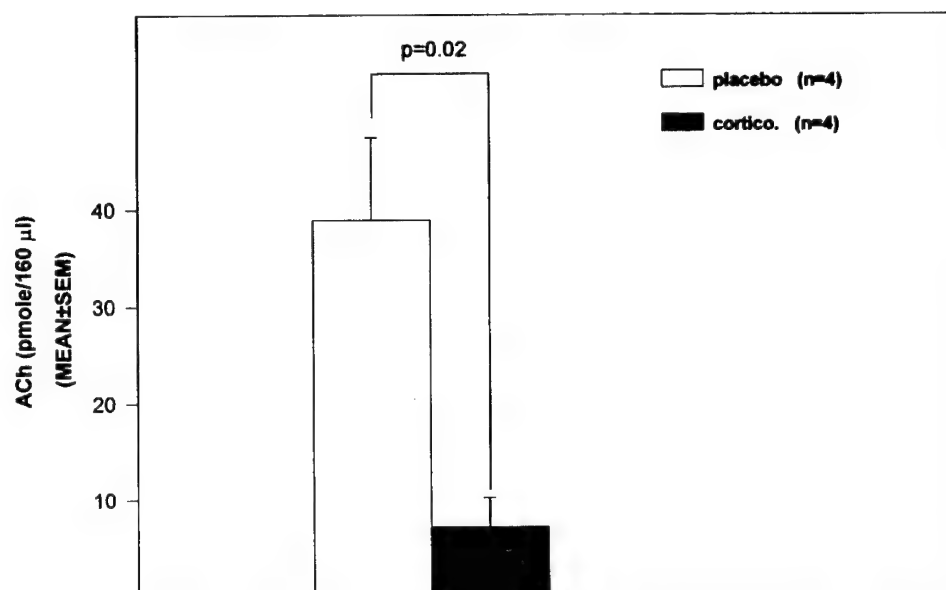


Figure 10.3 The effect of nine weeks corticosterone treatment on extracellular acetylcholine levels in the hippocampus, as measured by microdialysis in “nonimpaired” middle-aged rats, during 80 minutes after scopolamine injection (2 mg/kg ip), three months following termination of the corticosterone treatment.

the presynaptic feedback regulation results in a dramatic increase in extracellular ACh (Figure 10.3). Middle-aged “nonimpaired” rats were treated with CORT (or placebo) SR pellets for nine weeks. In the microdialysis study, a guide cannula was implanted in anesthetized rats using a stereotaxic instrument. The probe was inserted so that its 2mm membrane was placed at CA1/DG area (coordinates AP = -3.8 , $L = +1.6$ relative to bregma, $V = -1.7$ – -3.7). ACh was collected during 80 min following scopolamine injection (at a flow rate of 2 μ l/min), and measured by HPLC using an assay kit (MF-8910, BAS). The increase in the hormone-treated group was significantly lower compared to the placebo-treated group.

It is interesting to note that the microdialysis study was carried out three months following the high CORT treatment; thus, it probably represents permanent damage which might also lead to lasting cognitive impairments.

NIMODIPINE'S NEUROPROTECTION

The effects of corticosteroids on Ca homeostasis and their implications for neuroprotection and neurodegeneration were extensively discussed by Marian Jeols and her colleagues in the previous chapter of this book. The fact that GCs enhance Ca currents (Kerr *et al.*, 1992) and increase cytosolic Ca concentrations (Elliot and Sapolsky, 1991, 1993) seemed an important stage in the cascade of events leading to neurodegeneration. It had also been established that age increased GC-induced Ca

conductance (Kerr *et al.*, 1989). The same group recently reported that Ca influx in mammalian CA1 hippocampal neurons increased with age through the L-type voltage-activated channels (Thibault and Landfield, 1996). Several mechanisms may be involved in the change of neuronal Ca concentration (see Joëls in this book), but increasing evidence implies an important role to the L-type voltage-dependent channels in stress-aging interactions (e.g., see Kerr *et al.*, 1992). Some of the mechanisms suggested as involved in GCs' deleterious effects (e.g., glutamatergic cascade, see Sapolsky 1996a), may involve increased neuronal Ca at the final stage. The pharmacological interpretation of the above data led us to test the capability of the L-type Ca channel blocker, nimodipine, as neuroprotective drug against GC-induced "accelerated brain aging."

The design of the study was based on three-month old Fischer-344 rats, randomly divided into four experimental groups and treated for three months with two types of SR pellets:

- I Cort + nimodipine pellets
- II Cort + nimodipine-placebo pellets
- III Cort-placebo + nimodipine pellets
- IV Cort-placebo + nimodipine-placebo pellets

Nimodipine treatment affected neither the weight change nor CORT plasma levels of the two main treatments (CORT vs. placebo). Nevertheless, it had an exciting neuroprotective effect on hippocampal neurodegeneration (see Table 10.2). Recently, an additional double-blind study corroborated these light microscopy histological findings using EM examination (Beagley *et al.*, 1996).

Nimodipine was initially considered primarily a neuroprotective drug for subarachnoid hemorrhage and cerebral ischemia (Scriabine *et al.*, 1989). It was later found to enhance cognitive functions in aging rabbits (Deyo *et al.*, 1989) as well as in young rats, in which it also elevated hippocampal ACh (Levy *et al.*, 1991). Following numerous preclinical studies, nimodipine was also tested for its beneficial effects in human geriatric cognitive disorders (Schuurman and Traber, 1988; Ban *et al.*, 1990; Tollefson, 1990; Fritz and Walden, 1995).

The present findings extend the scope of neuroprotective action of Ca channel blockers to possible efficacy against stress-induced "accelerated brain aging". Thus, they supply additional pharmacological evidence to support the published association

Table 10.2 The protective effect of nimodipine treatment on corticosterone-induced hippocampal neurotoxicity

<i>Treatment</i>	<i>CA1 % of damaged cells</i>	<i>CA3 % of damaged cells</i>	<i>CA4 % of damaged cells</i>	<i>DG # of damaged cells</i>
Placebo/placebo	3.7 ± 1.4	3.1 ± 1.0	5.8 ± 3.2	7.7 ± 3.5
Placebo/nimodipine	2.2 ± 0.5	8.6 ± 4.1	7.1 ± 2.1	6.5 ± 1.7
Corticosterone/placebo	22.6 ± 7.9	22.7 ± 8.9	28.3 ± 8.4	51.0 ± 19.9
Corticosterone/nimodipine	3.2 ± 0.8	5.9 ± 2.9	2.1 ± 1.9	7.9 ± 2.8

between neurotoxic effects of GC and change in Ca homeostasis (Elliot and Sapolsky, 1993; Landfield and Eldridge, 1994).

IMPLICATIONS FOR HUMAN AGING

The relevance of animal studies to corticosteroid-brain interactions in humans has unfolded during the past few years. It was reported some years ago (Starkman *et al.*, 1992) that patients with Cushing's syndrome (tumor-induced high GC secretion) exhibited hippocampal atrophy which correlated reasonably with plasma cortisol levels. Urinary free cortisol was elevated in patients with dementia compared to a group of age-matched elderly patients (Maeda *et al.*, 1991). Aging itself affected cortisol levels and the response to stress (Raskind *et al.*, 1995). Sonia Lupien and her colleagues (1994) conducted a longitudinal study in 19 elderly subjects and found that those who had high cortisol levels that increased over the years were significantly impaired on explicit memory and selective attention tasks (Lupien *et al.*, 1994). In a continuation study of 51 healthy elderly volunteers, the evidence for subgroups was confirmed (Lupien *et al.*, 1996). Elevated levels of free cortisol were associated with impaired memory function in another study of healthy adults (Kirschbaum *et al.*, 1996).

Limited data is available on long term GC activation in humans. Prolonged prednisone treatment of patients with systemic disease, with no central nervous system involvement, was associated with impaired memory when compared with a control group of patients closely matched for age, sex, IQ and diagnosis (Keenan *et al.*, 1996). Vietnam combat veterans with post-traumatic stress-disorder (PTSD) showed deficits in verbal memory associated with smaller right hippocampal volume (Bremner *et al.*, 1996). In a recent review of human studies, Sapolsky (1996b) analyzed Bremner's combat stress data and found a high correlation between hippocampal volume and months of combat exposure.

The extensive animal studies, only partially reviewed in this chapter, and the recent human data suggest a connection between an environmental factor such as prolonged stress and accelerated brain aging. Our initial pharmacological findings suggest that future drug therapies might be able to counteract such deleterious actions.

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11 Stress Impairs Cognitive and Electrophysiological Measures of Hippocampal Function[☆]

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Throughout this century, studies have shown that the strength of the formation of memories is influenced by emotional state. In one of the earliest investigations of the relationship between arousal and cognition, Yerkes and Dodson (1908) demonstrated that the rate of learning declined with low or high levels of arousal. With an intermediate level of arousal, performance reached its optimal level. In the next decade, Stratton (1919) evaluated the complexity of the arousal-related modulation of memory. He discussed how emotions can produce either "hypermnestic" (enhancing) or "hypomnesic" (impairing) effects on learning. Subsequent analyses of the modulation of learning by arousal have extended these early observations to a broad range of species and tasks (Kirschbaum *et al.*, 1996; McGaugh, 1989). In recent years much work has been directed at understanding the neurobiological basis of the modulation of memory by behavioral state. In this chapter, we will present our work on the behavioral and hormonal modulation of the hippocampus, a temporal lobe structure necessary for learning and memory (Zola-Morgan and Squire, 1990). Our studies indicate that psychological stress exerts a transient impairment of hippocampal function, which is revealed both behaviorally as retrograde amnesia and physiologically as a blockade of synaptic plasticity.

THE HIPPOCAMPUS: DUAL ROLES IN STRESS AND LEARNING

A number of variables can influence whether increased emotionality enhances or impairs learning and memory, including the type of information to be learned, the

nature of the stressor, and the temporal relationship between the stressor and the learning experience (Christianson, 1992; Yerkes and Dodson, 1908). The changes in the brain that underlie the stress-induced modulation of memory are not well understood. It is known, however, that the hippocampus is involved in both memory and stress. First, research conducted during the last four decades has convincingly demonstrated that the hippocampus is critically involved in the storage of information. Damage to the hippocampus results in profound memory impairments in animals and people (Zola-Morgan and Squire, 1990). Second, the hippocampus is involved in the regulation of the hypothalamic-pituitary-adrenal (HPA) axis. Almost 30 years ago McEwen *et al.* (1968) found that the highest concentration of corticosterone taken up and bound by the brain was in the hippocampus. This finding helped to provide a context with which to understand studies showing that the hippocampus is involved in the regulation of stress responses (Dunn and Orr, 1984; Sapolsky *et al.*, 1991). Our goal, therefore, has been to develop a synthesis which will provide a greater understanding of the dynamics of the stress- and memory-related functions of the hippocampus.

One approach we have used is to study an electrophysiological model of memory under conditions of a stress challenge. The most widely studied physiological model of memory is a phenomenon referred to as long-term potentiation (LTP) (Barnes, 1995). LTP is an enhancement of synaptic transmission induced by high-frequency electrical stimulation of an afferent pathway. LTP occurs in a number of brain regions, but has been studied most extensively in the hippocampus. A vast amount of research supports the hypothesis that LTP is a physiological substrate of memory. For example, the development and decay of hippocampal LTP correlates with behaviorally assessed learning and forgetting. Moreover, pharmacological agents, such as NMDA receptor antagonists, selectively impair hippocampal-dependent learning and LTP (see Barnes, 1995 for review). Thus, studies have shown that: (1) the functional integrity of the hippocampus is necessary for normal learning to occur; and (2) hippocampal LTP is an electrophysiological model of memory, with mechanisms in common with memory formation.

Although LTP offers the prospect to study the biophysical mechanisms which underlie memory, the stimulation parameters commonly used to induce LTP are quite extensive (100–200 pulses/sec) and nonphysiological (i.e., hippocampal neurons do not fire 100–200 action potentials per second). The use of nonphysiological stimulation to induce LTP, therefore, left unclear the relevance of LTP to the encoding of behaviorally relevant information. My colleagues and I addressed this problem in our studies of a novel form of LTP, termed primed burst (PB) potentiation (Diamond *et al.*, 1988; Rose and Dunwiddie, 1986). This form of plasticity is induced by a pattern of electrical stimulation based on rhythmic electrophysiological activity (i.e., the theta rhythm), which occurs in the hippocampus of behaving rats (Bland, 1986). We found that only 5 pulses, delivered in a pattern of stimulation that mimicked the theta rhythm, induced a lasting enhancement in hippocampal synaptic transmission. Moreover, PB potentiation is more sensitive than is LTP to the modulatory effects of hormones and age (Diamond *et al.*, 1996b; Moore *et al.*, 1993b). The reduced threshold for generating PB potentiation, and the enhanced sensitivity of PB potentiation to behavior-related variables, suggests that patterned stimulation

activates mechanisms of plasticity that are similar to those that underlie memory formation.

**Given that Memory is Affected so Strongly by Emotionality,
How are LTP and PB Potentiation Affected by Stress?**

The first study to address this question was performed by Foy *et al.* (1987), who combined a behavioral analysis of LTP with *in vitro* electrophysiological recordings. These investigators used restraint and tailshock to stress rats and found that LTP was reduced in the hippocampus from stressed rats. In a complementary approach, we have studied the influence of behavioral state and hormones on the expression of hippocampal PB potentiation in behaving rats. We found that placing rats in an unfamiliar environment, which is a psychological stressor (Hennessy *et al.*, 1979), blocked PB potentiation (Figure 11.1) (Diamond *et al.*, 1990, 1994). These findings

Stress Blocks Primed Burst Potentiation

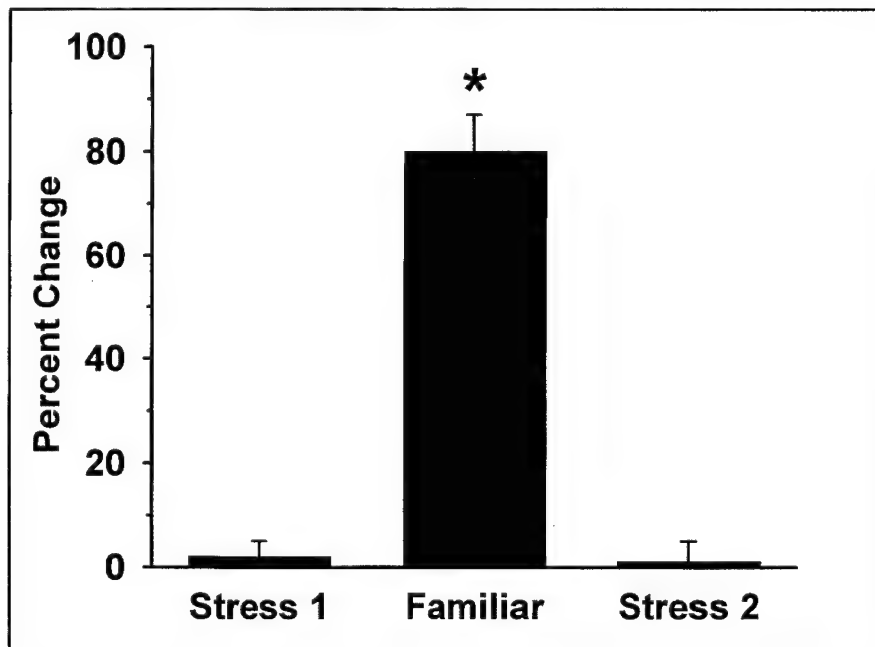


Figure 11.1 Primed burst (PB) stimulation of the hippocampal commissure produced an increase in the magnitude of the CA1 population spike only when the subjects were in a familiar environment. When naive rats were placed in the electrophysiological recording chamber ("Stress 1") PB potentiation did not occur. The subjects were then placed in the chamber every day for two weeks ("Familiar"), and a second PB stimulation was delivered. In the familiar environment PB potentiation occurred in every subject ($n=6$). Two weeks later, these same rats were placed in a second, distinctly different, recording chamber ("Stress 2"). Once again, PB potentiation was blocked in the unfamiliar environment. Elevated corticosterone levels occurred only when rats were placed in either of the two unfamiliar (stress) environments (Diamond *et al.*, 1994). * $p < 0.01$. These findings indicate that the induction of hippocampal plasticity is continually modulated by an animal's perception of its environment.

support the hypothesis that stress affects learning and memory by affecting PB- and LTP-like processes.

The hormonal basis of the stress-induced modulation of hippocampal LTP and PB potentiation appears to involve corticosterone. Foy *et al.* (1987) and Bennett *et al.* (1991) reported a negative correlation between elevated (stress) levels of corticosterone and the magnitude of LTP. In subsequent work, we showed that there is an inverted-U relationship between peripheral levels of corticosterone and the magnitude of PB potentiation (Diamond *et al.*, 1992). This finding has been further studied at the receptor level by Pavlides and his coworkers. These investigators reported that activation of hippocampal mineralocorticoid receptors, which are sensitive to low levels of corticosterone, enhanced plasticity. In contrast, activation of hippocampal glucocorticoid receptors, which are most sensitive to stress levels of corticosterone, blocked LTP (Pavlides *et al.*, 1995). These observations may be relevant to the well-described inverted-U relationship between stress and learning (Broadhurst, 1957).

FUNCTIONAL CONSEQUENCES OF THE STRESS-INDUCED BLOCKADE OF HIPPOCAMPAL PLASTICITY

The stress-induced impairment of PB potentiation and LTP suggests that stress should also impair cognitive measures of hippocampal function. However, empirical support for a direct connection between the effects of stress on hippocampal plasticity and on hippocampal-dependent memory has been difficult to obtain. For example, Shors and Dryver (1992) stressed rats prior to radial arm maze training and noted reduced rates of activity by the stressed subjects early in training. However, once the animals exhibited normal exploratory activity, their rate of learning was normal. In contrast, Luine and coworkers found that chronic pretraining stress affected performance in the radial arm maze. Whereas two weeks of pretraining restraint stress enhanced performance, three weeks of stress impaired performance (Luine *et al.*, 1994, 1996). The most direct test of the connection between the effects of stress on LTP and on hippocampal-dependent learning was performed by Warren *et al.* (1991). These investigators found that the same stress that been shown by others to block LTP, i.e., restraint and tailshock (Foy *et al.*, 1987), had no effect on hippocampal-dependent learning. Hence, the relevance of the stress-induced blockade of hippocampal plasticity, within the context of learning and hippocampal function, has been difficult to demonstrate.

Recently, my colleagues and I focused specifically on investigating how acute psychological stress affects memory (Diamond *et al.*, 1996c). The hypothesis guiding this work was that because stress blocked PB potentiation, it should also impair hippocampal-dependent memory. To test this hypothesis, we used a training paradigm that enabled us to evaluate the effects of stress on both hippocampal-dependent and hippocampal-independent memory. Food-deprived rats were trained on a 14-arm radial maze. Seven of the arms were never baited (i.e., never contained food), whereas the other seven were baited with a single pellet. Over the course of a month of daily training, the rats optimized their foraging strategy in the following manner: (1) they entered each of the seven baited arms a single time, ate the food, and then

did not return to those arms during that trial; and (2) they avoided entering the seven arms that never contained food.

This training procedure enabled us to test two distinct memory systems. The first memory system was "working memory", which is a *hippocampal-dependent* form of memory (Olton *et al.*, 1979). Working memory information changes with each training trial. The working memory component of the task was that once the rat ate the food in an arm, it needed to avoid returning to that arm because it no longer contained food. A reentry into an arm that was depleted of food was a working memory error. The second memory system was "reference memory", which is a *hippocampal-independent* form of memory (Olton *et al.*, 1979). Reference memory information is constant across all trials. The reference memory component of this task was that the rats remembered that seven of the arms never contained food. An entry into an arm that never contained food was a reference memory error.

When the rats were allowed to move through the maze without interruption they efficiently located all 7 food pellets without making working or reference memory errors. To make the task more difficult we interposed a 4-hour delay period between the 4th and 5th baited arm selections. That is, the subjects were first allowed to enter and eat the food in 4 of the 7 baited arms. After eating the 4th pellet, they were removed from the maze and placed either in a familiar environment (their home cage) or in an unfamiliar environment (an electrophysiological recording chamber) for four hours. At the end of the 4-hour delay period they were returned to the maze. In the postdelay period, rats had to avoid the 7 arms that were never baited (reference memory test), and also avoid entering the 4 arms that they had entered prior to the delay period (working memory test). To perform optimally the rats needed to enter only the 3 remaining baited arms.

Placement of the subjects in their home cage during the delay had no significant effect on either their working or reference memory. The stress manipulation, by contrast, produced a significant and selective effect on their memory. When the rats were returned to the maze after being in the unfamiliar environment, their working memory was severely impaired (Figure 11.2, right). Reference memory, on the other hand, was unaffected by the stress manipulation (Figure 11.2, left). Thus, psychological stress produced retrograde amnesia; when the subjects were stressed they were unable to remember which of the baited arms they had entered before the delay phase (working memory impaired), but they had no difficulty avoiding the arms that were never baited (reference memory intact).

Our finding of a selective impairment of working memory is consistent with previous work that showed a transient disruption of hippocampal function, either by pharmacological means or by electrical stimulation, produced retrograde amnesia for working memory (Maki, 1986; Mizumori *et al.*, 1985). Here, we have shown that hippocampal function was disrupted solely through the use of the psychological stress of exposure to an unfamiliar, and unavoidable, environment.

Are the Experimental Findings Applicable to Cognitive Challenges Faced by People?

We would suggest that the working/reference memory distinction, and the selective susceptibility of working memory to stress, is relevant to experiences encountered by

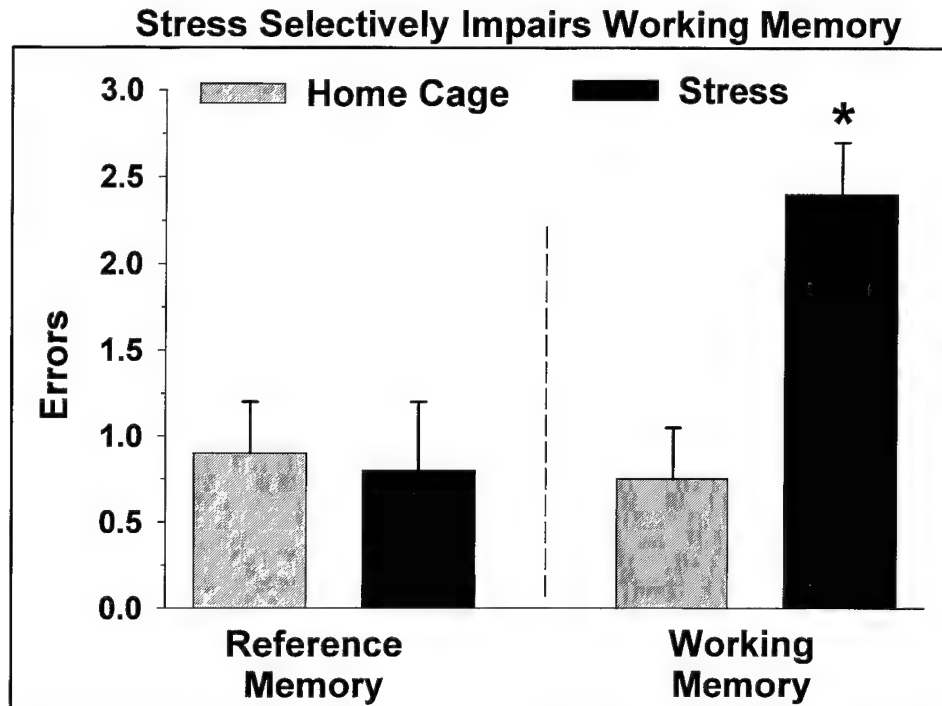


Figure 11.2 Exposure to an unfamiliar environment (the electrophysiological recording chamber) selectively interfered with spatial working memory. When the rats ($n=12$) were stressed during the delay period (placed in the recording chamber) the number of reference memory errors was not significantly affected (left side), but working memory errors increased significantly (right side). Data represent the mean number of errors when the subjects were placed in their home cage compared to the first two days in which they were exposed to the stress environment. See Diamond *et al.* (1996c) for additional details concerning the habituation of this effect, as well as the reappearance of the memory impairment when a second stressor was used. * $p < 0.05$.

people in daily life. For example, finding one's car is both a working and reference memory task if the car is left in a different location each day. In this case, a daily "trial" consists of two components: (1) remembering the place where the car was last parked (working memory; dynamic information); and (2) remembering the appearance of the car (reference memory; stable information).

In theory, hippocampal-dependent (working) memory in people should be more susceptible to disruption by stress than should be reference memory. Indeed, rigorous tests of this theory have shown that hippocampal-specific memory is more susceptible to impairment by stress and stress hormones than is nonhippocampal memory. Studies in rats (Rudy, 1996) and people (Kirschbaum *et al.*, 1996) have shown that acute psychological stress selectively interferes with hippocampal-dependent, but not hippocampal-independent, memory. Moreover, chronic stress or corticosterone administration can impair hippocampal-dependent spatial learning in rats (Bodnoff *et al.*, 1995; Dachir *et al.*, 1993; Luine *et al.*, 1994), and cortisol or cortisol agonists impair hippocampal-dependent memory in people (Kirschbaum

et al., 1996; Newcomer *et al.*, 1994; Rubinow *et al.*, 1984). Finally, performance in hippocampal-independent tasks, including some forms of classical conditioning and motor learning, was not impaired by stress (see Diamond *et al.*, 1996c). Overall, the findings indicate that, across species, stress selectively disrupts hippocampal function.

NEUROSTEROIDS AS ANTISTRESS HORMONES

Our work indicates that behavioral stress and elevated levels of corticosterone impair hippocampal function in rats. It is also known, however, that stress or arousal can enhance memory. For example, traumatic events, such as combat experience or physical attack, can produce such strong memories that they become pathological and intrusive, interfering with learning and memory years after the original experience (Bremner *et al.*, 1993). Similarly, highly arousing, but nonthreatening, experiences can enhance memory (Cahill *et al.*, 1994). The challenge, which has yet to be sufficiently addressed empirically, is to understand how stress can strengthen, as well as impair, the formation of memories.

A recently described category of steroids, termed neurosteroids (Baulieu and Robel, 1990), may provide insight into the complexity of the effects of stress on cognition. These hormones are produced, *de novo*, in the periphery and also in the brain. One particular type of neurosteroid, dehydroepiandrosterone sulfate (DHEAS), is the most abundant adrenal steroid produced in humans (Baulieu and Robel, 1990; Kalimi *et al.*, 1994). DHEAS enhances cell survival, stimulates immune function, increases neural excitability, and improves memory (Frye and Sturgis, 1995; Kalimi *et al.*, 1994). These characteristics of DHEAS contrast with corticosterone, which has largely catabolic effects on physiology and suppressive effects on immune function. In general, DHEAS has properties that are so consistently antagonistic to those of corticosterone that it has been described as an antigluccorticoid hormone (Kalimi *et al.*, 1994). The complex effects of stress on cognition may be due, in part, to a competitive interaction between corticosterone and DHEAS.

We have begun to address how DHEAS affects electrophysiological and cognitive measures of hippocampal function. Our initial work has shown that there is an inverted-U enhancement of PB potentiation by DHEAS (Diamond *et al.*, 1996b), which can be blocked by stress (Diamond *et al.*, 1995). We also evaluated the possibility that DHEAS would enhance hippocampal-dependent learning. Rats were administered DHEAS and then trained in the Morris water maze, a spatial learning task that tests hippocampal-dependent memory. The subjects given DHEAS developed an inverted-U enhancement between the dose of DHEAS and behavioral performance; that is, intermediate doses of the hormone were the most effective at enhancing memory (Diamond *et al.*, 1996a). Thus, administration of exogenous DHEAS enhanced both electrophysiological and cognitive measures of hippocampal function.

SUMMARY

Heightened emotionality affects the strength of the formation of memories. We have studied the influence of behavioral state and stress hormones on hippocampal function from cognitive and electrophysiological perspectives. We found that psychological stress blocked the induction of primed burst (PB) potentiation, an electrophysiological model of memory. PB potentiation was also affected by stress hormones. Specifically, elevated (stress) levels of corticosterone, an adrenal steroid released during stress, blocked PB potentiation; however, relatively low (nonstress) levels of corticosterone enhanced PB potentiation. Overall, there was an inverted-U relationship between corticosterone levels and PB potentiation. We also studied the effects of administration of the neurosteroid dehydroepiandrosterone sulfate (DHEAS) on hippocampal electrophysiology and spatial learning. There was an inverted-U function between the dose of DHEAS administered and the magnitude of PB potentiation, as well as an inverted-U enhancement of spatial memory by DHEAS. Therefore, the capacity for stress to enhance or impair memory may be due, in part, to a competitive interaction between corticosterone and DHEAS. In behavioral work, we found that stress selectively impaired hippocampal-dependent, but not hippocampal-independent, spatial memory. This finding is consistent with other work demonstrating that there is a selective susceptibility of hippocampal-dependent memory to be impaired by stress in people. Thus, the vulnerability of the hippocampus to be disrupted by stress is conserved across species.

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III. Stress and Development

12 The Developmental Neurobiology of the Response to Stress: Multiple Levels of Corticotropin Releasing Hormone Regulation

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The ability to respond to adverse environmental cues is present in the neonatal and infant rat, although in an immature form. A number of laboratories have demonstrated stress-induced elevations of plasma glucocorticoids (GCs) during the first two postnatal weeks. However, the limbic and hypothalamic mechanisms that control the hormonal stress-response during this period, and the nature of the differences in the regulation of the immature and adult stress response are not fully understood.

Both hypothalamic corticotropin releasing hormone (CRH) and vasopressin contribute to adrenocorticotropin hormone (ACTH) release from the pituitary in the adult. The relative roles of these two peptides during the neonatal (first week) and infant (second week) developmental period are controversial. Evidence is presented that argues strongly for a major role for CRH.

CRH messenger-ribonucleic acid (CRH-mRNA) is first detectable in the hypothalamic paraventricular nucleus (PVN) on the 17th fetal day in the rat. The onset of CRH synthesis and the levels of CRH-mRNA are not influenced by GC in the fetal and neonatal rat. Further, upregulation of hypothalamic CRH synthesis is a major component in the mature stress response. CRH-mRNA levels in the PVN are increased with cold stress by the ninth postnatal day, but not during the first postnatal week.

CRH-mediated neurotransmission in both the endocrine and neuronal effector arms of the response to stress may be modulated via alteration of receptor number. The first member of the CRH receptor family, CRF₁, likely mediates the neuroendocrine effects of CRH. The developmental profile of CRF₁-mRNA reveals regional, age-specific control of the synthesis of this receptor. Receptor expression profile may provide important information regarding modulation of the age-specific roles of CRH in different regions. For example, a high ratio of hippocampus/amygdala receptors may preferentially activate negative hippocampal input to the hypothalamus during the neonatal period. Additionally, increased CRH receptor mRNA in the infant compared with the adult provides a mechanism for enhanced excitatory effects of the peptide during this age.

In summary, there is increasing evidence for multiple control points of the early postnatal response and adaptation to stress. CRH synthesis in the hypothalamus and amygdala, its sensitivity to GC feedback, and the abundance and distribution of at least two distinct CRH receptors in the limbic CNS and the pituitary are developmentally regulated. All serve as control points permitting an effective endocrine, autonomic and behavioral response to stressful environmental cues.

INTRODUCTION

The ability to respond and adapt to environmental cues is fundamental for survival of all complex organisms. The brain-adrenal system is a major effector of the mammalian response to stress (Sawchenko *et al.*, 1993). The neuroendocrine component of this response consists of a complex, regulated increase in the synthesis and secretion of a number of hormones (Dallman *et al.*, 1987). Upon impact of a stress signal into the limbic system, particularly the central nucleus of the amygdala, activation of the hypothalamic PVN leads to secretion of CRH into the hypothalamic-pituitary portal system (Vale *et al.*, 1983). Activation of CRH receptors in the pituitary by the peptide leads to release of ACTH, which acts on the adrenal to promote GC secretion. Negative feedback loops at multiple levels, such as the hippocampus and hypothalamus, then result in a "shut-off" of this stress-induced activation of the hypothalamic-pituitary-adrenal (HPA) system. In addition to, and likely as a result of CRH release from the PVN, increased CRH gene expression occurs within hours of the acute stress, so that messenger RNA levels of CRH in the PVN in animals exposed to acute stress are higher 2–4 hours later as compared with nonstressed controls (Lightman and Harbuz, 1993; Yi and Baram, 1994; Makino *et al.*, 1995).

The developmental onset of the hormonal response to stress has been an active topic of investigation. Quantitative and potentially qualitative differences from the mature stress response have been described during the first two postnatal weeks, leading to the term "stress-hyporesponsive period" (Sapolsky and Meaney, 1986). More recently, stress-induced elevations of plasma corticosteroids during the first two postnatal weeks has been demonstrated by several groups (e.g., Walker *et al.*, 1986, 1991; Avishai-Eliner *et al.*, 1995). The limbic and hypothalamic mechanisms controlling the stress response during this period are not fully understood. In this report we focus on the role of CRH in the response to acute and chronic stress during the first two postnatal weeks in the rat. We provide evidence that CRH secretion occurs in response to stress and determines plasma corticosterone levels. However, CRH gene expression is not significantly influenced by stress or GC levels during the first postnatal week. These results suggest a fundamental qualitative difference between the regulation of the CRH gene promoter in the neonatal and mature rat.

METHODOLOGICAL ISSUES IN THE STUDY OF THE STRESS-RESPONSE OF THE IMMATURE RAT

Choice of Stress Paradigm

The hormonal response to stress is determined to some extent by the stress paradigm used. Prototypical stress paradigms in the adult have included acute footshock or restraint (Rivest *et al.*, 1995). In the adult rat, immobilization is a far more powerful stress than cold exposure (Harbuz and Lightman, 1989). This is probably due to the presence of fur and of a fully mature thermoregulation. In the infant rat, exposure to cold constitutes a powerful age-specific stress, due to lack of fur and

immature thermoregulation (Yi and Baram, 1994). Similarly, separation from the mother constitutes a unique, age-specific stress to the rat pups, and separation provokes an enhanced response to acute stressors, such as cold (Sucheki *et al.*, 1993). However, significant differences in the magnitude of plasma corticosterone elevation is evident among rats separated from their mother as a group (G-DEP) as compared to those separated from both mother and siblings (I-DEP in Figure 12.1; Avishai-Eliner *et al.*, 1995).

The Importance of Baseline Plasma Corticosterone

Plasma hormone level elevations in response to stress are compared to baseline levels. Clearly, the magnitude of the observed stress-induced elevation (i.e. percent or fold increase, or absolute increment) is dependent on the plasma corticosterone level determined in the nonstressed state. We found that removal from the home cage for as little as two minutes results in significant plasma corticosterone elevation in infant rats (1.5–3.5 µg/dl) as compared to pups sacrificed within 45 seconds of the initial disturbance. Indeed, baseline plasma corticosterone levels in the studies of the stress response in neonatal and infant rats summarized by Sapolsky and Meaney (1986) were mostly 3–10 µg/dl. The conclusion from these studies was that there was, at most, a blunted stress-induced augmentation of corticosterone secretion. Similarly, Levine (1970) reported baseline levels of 10 µg/dl in 6–12-day-old pups, with little change after electric shock. Conversely, Walker and colleagues (1991) documented a robust and stressor-specific plasma corticosterone response to a number of acute stresses in 5 and 10-day-old rat pups. The baseline, nonstressed values for plasma corticosterone in these studies were less than 1 µg/dl.

In a study of the hormonal effects of exposure to acute cold stress, we documented a substantial elevation of plasma corticosterone in the 4, 6, 9, and 12-day-old rat, that is, throughout the first two postnatal weeks (Yi and Baram, 1994). The baseline values of nonstressed pups were between 0.7 and 1.8 µg/dl. As is shown in Figure 12.2, stress increased plasma corticosterone 470% on postnatal day 6, 345% on postnatal day 9 and 564% on postnatal day 11–13 (Yi and Baram, 1994).

The Importance of Sampling Time

In the time-course study of the hormonal stress response in the neonatal and infant rat (Yi and Baram, 1994), plasma corticosterone elevations peaked 60 minutes after the termination of cold stress. On many occasions, a two- to three-fold increase was found between the 40 and 60 minute samples. Similar results were reported by Walker *et al.* (1991) for histamine injection stress. However, a number of studies (e.g., Levine, 1970) sampled corticosterone only up to 30 minutes after stress termination, a procedure that would miss the peak of corticosterone levels. Walker and colleagues, reporting on the effects of cold stress, described maximal plasma of corticosterone three hours after stress termination; however, they did not rewarm the pups (Walker, CD, personal communication), whereas Yi and Baram did.

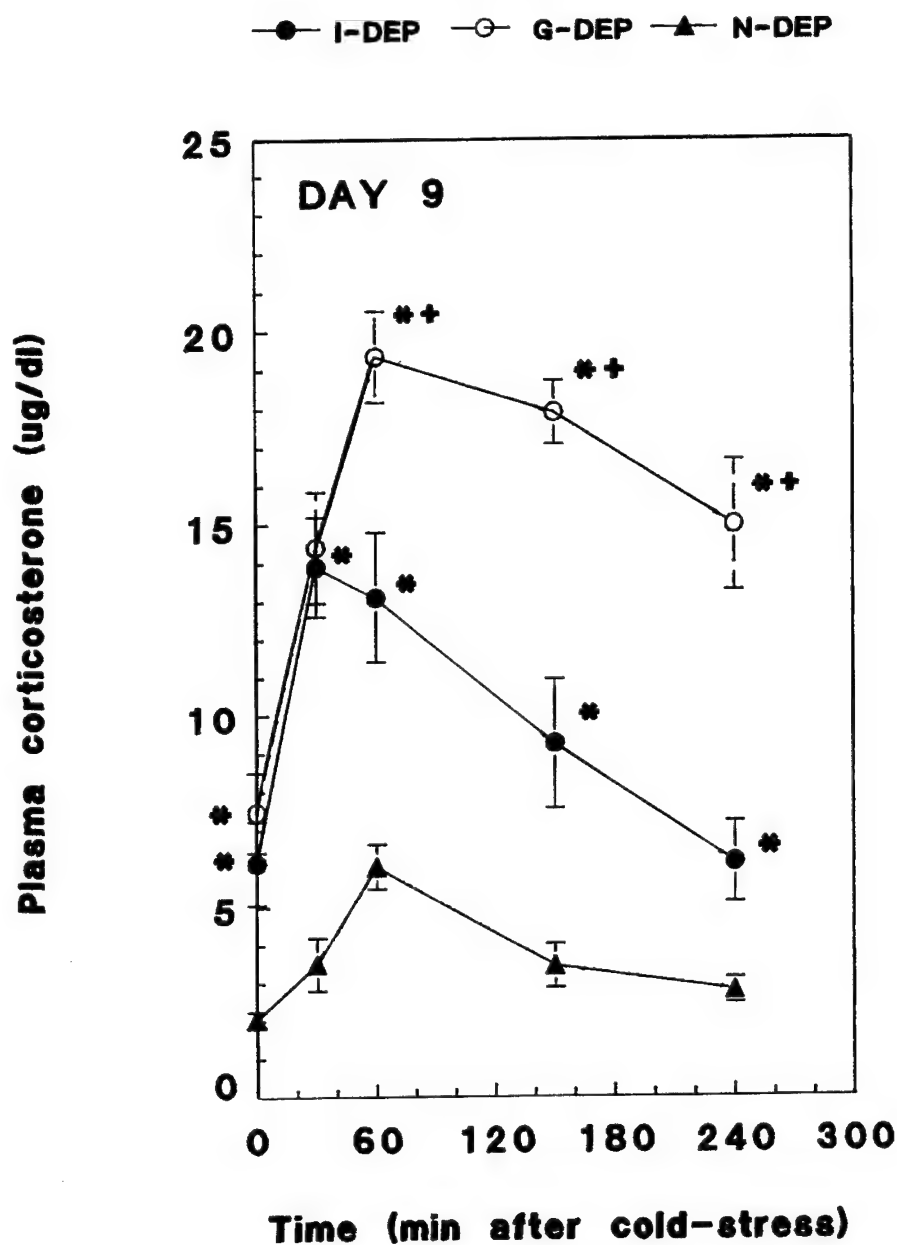


Figure 12.1 Time-course of plasma corticosterone elevation in response to cold-stress in nine-day-old rats after a 24 hour maternal deprivation period. N-DEP rats were kept in bedded cages with their mothers for the 24 hours prior to cold exposure (60 minutes). G-DEP rats were kept in a bedded, euthermic cage as a group. I-DEP rats were kept normothermic, but were isolated from both their mother and their siblings, and probably could not hear supersonic calls. Plasma corticosterone of both groups of maternally deprived pups were significantly higher than those of nonseparated pups at all time points (* $p < 0.05$). Grouped maternally, deprived pups (G-DEP) had significantly higher plasma corticosterone as compared with the I-DEP experimental group (+ $p < 0.05$). (Modified from Avishai-Eliner *et al.*, 1995; with permission.)

Effect of cold-stress on plasma corticosterone in neonatal rats

△—△ Day 11–13 ○—○ Day 4–6 ▲—▲ Day 2–3

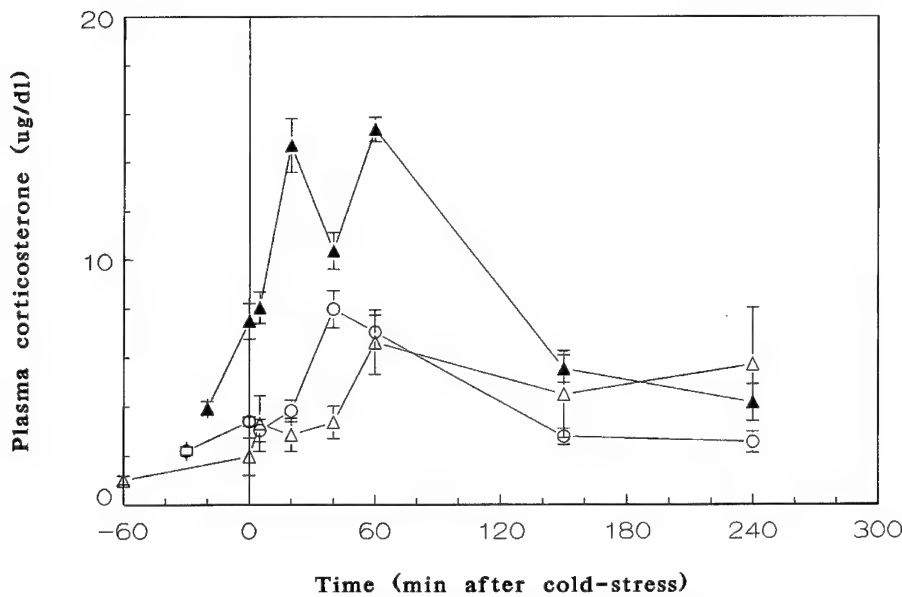


Figure 12.2 A composite time-course representation of plasma corticosterone induced by cold-separation stress in 2–3, 4–6 and 11–13-day-old rats. Time 0 denotes the end of the maximally tolerated cold exposure (Yi and Baram, 1994). Thus, nonstressed levels were obtained 30–60 minutes earlier, at the time of initiation of cold exposure. All age groups had at least a four-fold increase of plasma corticosterone after the acute cold stress.

PLASMA ACTH AND CORTICOSTERONE INDUCTION BY ACUTE COLD STRESS DEPENDS ON CIRCULATING CRH

Both hypothalamic CRH and vasopressin mediate ACTH release from the pituitary. The relative roles of these two peptides during the neonatal (first week) and infant (second week) developmental period is controversial (Muret *et al.*, 1992). Using acute cold-separation stress, the input of CRH and AVP to plasma ACTH and corticosterone elevation was investigated in both the neonate (six-day old) and infant (nine-day old) rat. Rat pups were injected with either normal rabbit serum (as control) or an antiserum to CRH (courtesy Dr. W.W. Vale). Immunoneutralization of CRH entirely eliminated the cold-separation-induced elevation of plasma ACTH (Figure 12.3) and corticosterone (Yi and Baram, 1994). The injection procedure itself, whether of normal serum or antiserum, led to an elevation of plasma ACTH and corticosterone levels that was not sensitive to anti-CRH. This may be due to the fact that pituitary receptors to CRH were already activated by the time the antiserum reached the portal circulation, or to a different, CRH-independent mechanism for

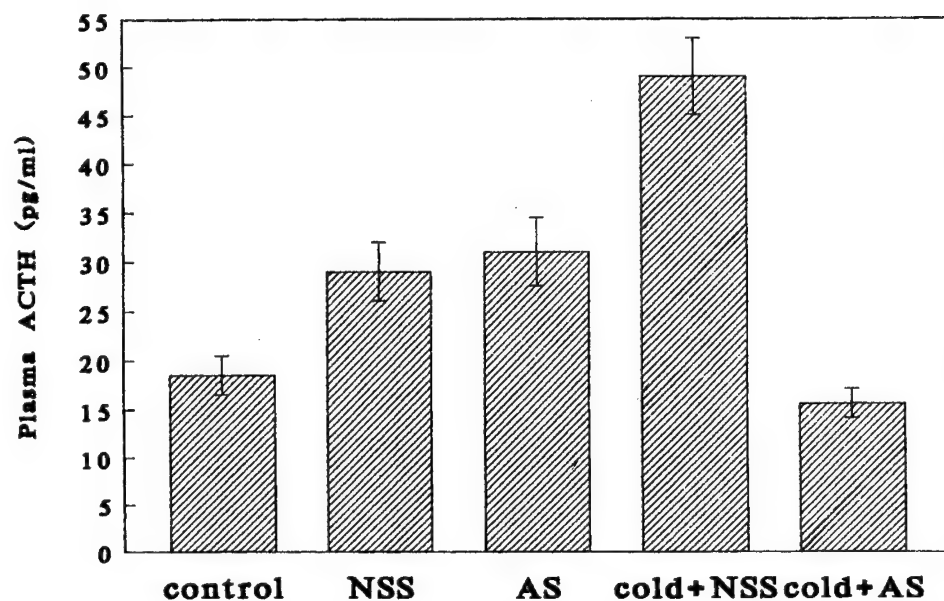


Figure 12.3 Effect of passive immunization against CRH on cold stress-induced plasma ACTH elevation in 6-day-old rats. Values are the mean \pm SEM of 4–6 rats. NSS = normal sheep serum, AS = antiserum directed against CRH. *Significantly different from NSS + cold ($p < 0.05$).

pain-induced elevation of plasma ACTH and corticosterone. In conclusion, an age-specific acute stress, cold-separation, activates the CRH-ACTH-corticosterone axis, resulting in robust elevations of plasma corticosterone in the neonatal and infant rat.

THE DEVELOPMENTAL PROFILE AND THE REGULATION OF CRH SYNTHESIS IN THE PVN

Using *in situ* hybridization, CRH-mRNA is first detectable in the PVN of the rat on the 17th fetal day (Baram and Lerner, 1991; Grino *et al.*, 1989a), though CRH-like immunoreactive cells have been described earlier (Bugnon *et al.*, 1982; Daikoku and Hisano, 1992) and CRH-mRNA is visible on northern blots of fetal rat brain as early as the 15th fetal day (Emmanuel *et al.*, 1989). CRH gene expression is robust during fetal days 18 and 19, but is markedly decreased prior to birth (fetal days 20 and 21, Baram and Lerner, 1991; Grino *et al.*, 1989a). CRH-mRNA levels remain quite low during the first postnatal days, then increase to reach adult levels by the end of the first postnatal week.

In order to study whether CRH synthesis during fetal life is regulated by GCs (Chao *et al.*, 1989; Herman *et al.*, 1989; Imaki *et al.*, 1991), plasma corticosterone levels were manipulated using a “pharmacological adrenalectomy” paradigm (Plotsky and Sawchenko, 1987). Despite drastic reduction of plasma GC levels in the

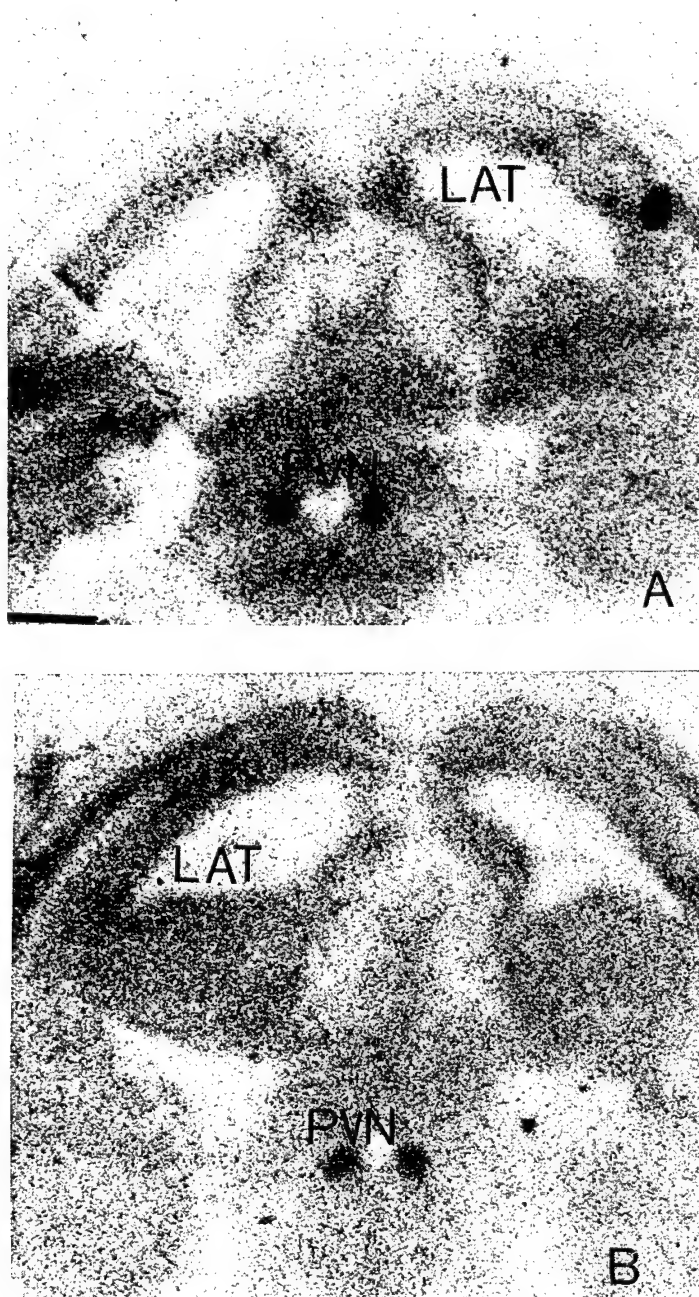


Figure 12.4 Coronal sections of the brains of 18th day fetal rats, at the level of the paraventricular nucleus (PVN) of the hypothalamus. Sections were subjected to *in situ* hybridization using a deoxy-oligonucleotide probe for the CRH messenger RNA. Hybridization signal is evident over the globular PVNs. CRH-mRNA levels in the control rats (B) did not differ from those in a rat subjected to pharmacological adrenalectomy in utero (A). LAT = lateral ventricle. Bar = 0.7 mm. (From Baram and Schultz, 1992, with permission.)

fetus (Baram and Schultz, 1990; Baram and Schultz, 1992) and an increase in CRH-mRNA abundance in pregnant adult rats, CRH gene expression in the fetal hypothalamus was not altered (Figure 12.4). Further, the onset of *in situ* hybridization-detectable CRH-mRNA, on the morning of the 17th fetal day, was not found to occur earlier in the face of low GC levels (Baram and Schultz, 1992). The lack of alteration of CRH-mRNA levels by GC in the fetal rat is not due to absence of GC receptors. GC receptor mRNA has been shown in the PVN as early as the 16th fetal day (Yi *et al.*, 1994). All of these findings support other experimental evidence for altered function of the negative GC feedback portion of the HPA loop in the perinatal rat (Levine, 1970; Walker *et al.*, 1986; Grino *et al.*, 1989).

In conclusion, CRH gene expression in the PVN of the fetal, neonatal and infant rat reveals a unique developmental profile. Furthermore, GCs do not seem to regulate the onset of CRH gene expression or the levels of CRH-mRNA in the fetal rat PVN.

STRESS-INDUCED UP-REGULATION OF CRH GENE EXPRESSION IS DEFICIENT DURING THE FIRST POSTNATAL WEEK

Upregulation of hypothalamic CRH synthesis is a major component in the mature stress response. Stress leads to depletion of hypothalamic stores of CRH (Ixart *et al.*, 1987) and to a "compensatory" increase of CRH-mRNA levels in the PVN (Lightman and Harbuz, 1993). As discussed above, CRH is secreted in response to stress throughout the first two postnatal weeks, leading to elevation of plasma corticosterone. However, as is seen in Figure 12.5, there is no upregulation of steady state CRH-mRNA levels in the PVN of the six day old rat. By the ninth postnatal day, stress is capable of increasing CRH synthesis, as measured by enhancement of CRH-mRNA levels in the hypothalamic PVN (Yi and Baram, 1994).

BLOCKING GC RECEPTORS IN THE PVN "DISINHIBITS" CRH GENE EXPRESSION ONLY IN RATS OLDER THAN NINE DAYS

In the mature animal, hypothalamic levels of CRH are regulated by GCs (Jingami *et al.*, 1985). To a large extent, CRH-mRNA levels in the PVN are determined by GCs acting via local GC receptors in the hypothalamus (Swanson and Simmons, 1989).

During the first postnatal week, both plasma GC levels and CRH-mRNA in the PVN are low (see above). This covariance of CRH-mRNA and plasma GCs rules out a simple negative feedback control at this age. The control of CRH-mRNA levels by GCs was examined during the first two postnatal weeks, via chronic implantation of a stainless steel cannula containing a specific GC-receptor antagonist (RU-38486; Moguilewsky and Philibert, 1984). The cannula was placed immediately above the PVN; precise calculations permitted estimation of the amount of released antagonist over three days (Yi *et al.*, 1993; Yi and Baram, 1993). Pharmacological blocking of the receptor did not "disinhibit" CRH-gene expression during the first postnatal week, but upregulated PVN-CRH-mRNA starting on days 9–12. As is the case in the fetal

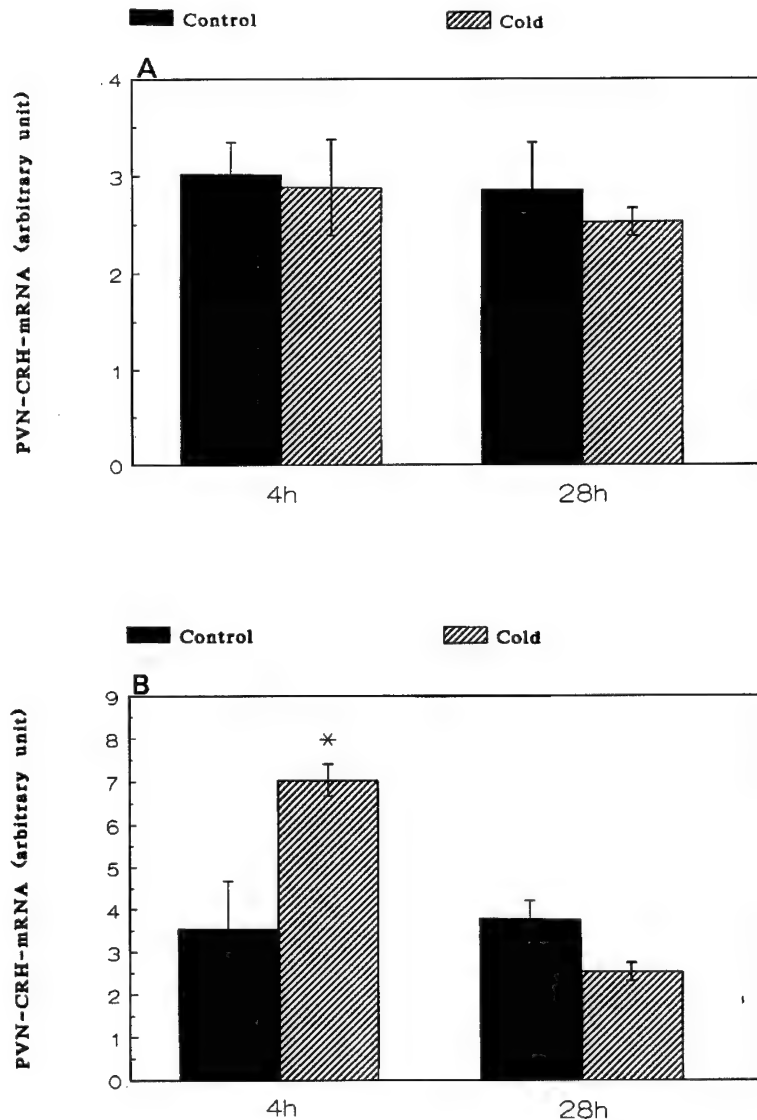


Figure 12.5 Effect of cold-separation stress on the levels of CRH-mRNA in the paraventricular nucleus (PVN) of 6-day-old (top) and 9-day-old (bottom) rats. Pups were subjected to age-appropriate maximal tolerated cold-stress (Yi and Baram, 1994). CRH-mRNA was determined using *in situ* hybridization. Values denote mean \pm Standard errors. *Significantly different from control ($p < 0.05$).

rat, the lack of CRH-mRNA increase in RU-38486 treated pups was not due to the absence of GC receptors. We, and others, documented abundant GR-mRNA in the PVN as early as the 16th fetal day (F16), prior to the onset of CRH gene expression in the PVN (Yi *et al.*, 1994). GC-receptor-mRNA in hippocampus (mainly CA1 and CA2) and PVN was robust throughout the late fetal and neonatal period.

Taken together, these findings demonstrate that despite the presence of abundant receptors, GCs do not downregulate CRH synthesis on the first postnatal week. This is fundamentally different than the well-documented negative feedback of GCs on CRH synthesis in adults.

THE DEVELOPMENTAL PROFILE OF THE RECEPTOR FOR CRH IN LIMBIC REGIONS OF THE RAT BRAIN

CRH-mediated neurotransmission, in both the endocrine and neuronal effector arms of the response to stress, may be modulated via alteration of receptor number (De Souza *et al.*, 1985; Rivest *et al.*, 1995). The first member of the CRH receptor

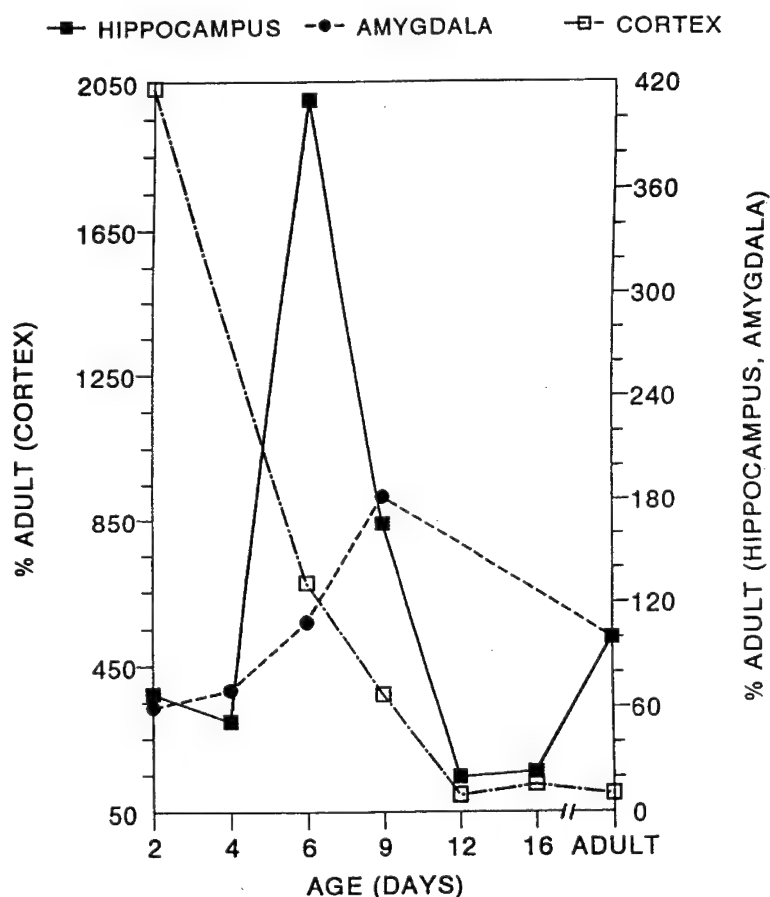


Figure 12.6 Diagram showing the relative levels of messenger RNA for the CRH receptor, CRF, in the hippocampal ammon's horn, the central and lateral amygdala nuclei and the frontoparietal cortex as a function of age in the rat. The distinctive developmental profile of this receptor is evident (modified from Avishai-Eliner *et al.*, 1996, with permission).

family, CRF₁, has been demonstrated in brain, pituitary and other organs, and probably mediates the neuroendocrine effects of CRH (Chang *et al.*, 1993; Perrin *et al.*, 1993). CRF₁-mRNA distribution during development reveals several distinctive spatial and temporal patterns (Avishai-Eliner *et al.*, 1996). In hippocampal CA1, CA2 and CA3a, maximal (300–600% adult) CRF₁-mRNA levels are found on postnatal day 6. In the amygdala, CRH receptor mRNA levels peak on the ninth postnatal day (at 180% of adult values). In the fronto-parietal cortex, a steady decline from high postnatal day 2 levels results in adult levels by day 12. These findings demonstrate a distinct, regional, age-specific control of the synthesis of CRF₁ (Figure 12.6). A second member of the CRH receptor family CRF₂, found primarily in the brain, has recently been defined (Lovenberg *et al.*, 1995). The developmental profile of CRF₂-mRNA may offer an additional control point for potential age-specific effects of CRH.

The developmental pattern of the expression of CRH receptors provide important information regarding modulation of age-specific roles of CRH in different brain regions. For example, a high ratio of hippocampus : amygdala receptors may preferentially activate negative hippocampal input into the PVN during the neonatal period (Jacobson and Sapolsky, 1991). Increased CRH receptor mRNA in the infant compared with the adult also provides a mechanism for the high excitatory effect of the peptide at this age (Ehlers *et al.*, 1983; Baram and Schultz, 1991; Baram *et al.*, 1992).

CONCLUSION

In summary, there is increasing evidence for multiple control points for modulating the early postnatal response and adaptation to stress. CRH secretion and synthesis in the hypothalamus and amygdala, and the abundance and distribution of at least two distinct CRH receptors in the limbic CNS and the pituitary may all be altered by environmental cues, permitting effective GC secretion even during the “stress-hyporesponsive” developmental period.

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13 Long-Term Influences of Perinatal Life Events on Behavioral and Biological Responses to Stimuli: Role of the Mother

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In humans, prenatal stress can induce mental retardation and sleep disturbances in infants. In animals, dams stressed during pregnancy can bear offspring with reduced male sexual activity, enhanced emotional reactivity, increased propensity to self-administer drugs, and modifications of glucocorticoid secretion. We studied stress-induced corticosterone (CORT) secretion and hippocampal CORT receptors in adult male rats submitted to prenatal stress (PS). Repeated restraint of the dam during the last week of pregnancy has been used as PS. At 90 days of age, offspring were submitted to a 30-min restraint stress. We found that PS decreases hippocampal CORT receptors and prolongs stress-induced CORT secretion in adult rats.

Then we tested if the stress-induced increase in maternal glucocorticoids, which cross the placental and blood-brain barriers reaching the foetus, may play a role. We investigated the effects of adrenalectomy and/or CORT injections to the mother on the outcome of PS on the activity of the hypothalamo-pituitary-adrenal (HPA) axis of adult male offspring. We found that: (1) maternal adrenalectomy protected the offspring from the effects of PS on CORT-secretion receptors; and (2) injections of CORT to the mother had the same effects of PS.

Prenatal and postnatal events affect different behaviors, but can also impinge differently on the same behavioral response, such that postnatal manipulations can reverse the behavioral effects of prenatal stress. For example, postnatal handling has been shown to reverse the increase in emotional reactivity induced by prenatal stress. To this end, we tested the interactions of prenatal and postnatal events on the activity of the HPA axis. Adoption at birth was used to perturb the postnatal environment. We found that: (1) adoption, independently of the stress experience of the foster mother, reverses the effects of prenatal stress on both corticosterone-secretion and corticosteroid receptors; (2) adoption *per se* increases maternal behavior and decreases the stress-induced corticosterone secretion peak in adult offspring.

In conclusion, prenatal and postnatal manipulations can have opposite long-term effects on the activity of the HPA axis and adoption, probably by changing maternal behavior can protect against the effects of prenatal stress. Thus, changes in the activity of the hypothalamo-pituitary-adrenal axis may be one of the biological substrates of the long term effects of perinatal events.

INFLUENCE OF PERINATAL ENVIRONMENT CHANGES ON ORGANISMAL DEVELOPMENT

Changes of prenatal and postnatal environments exercise complex influences on the development of an organism. In particular, life events occurring during those two

early periods of life can have different long-term behavioral effects. For example, prenatal stress can induce mental retardation and sleep disturbances in human infants (Stott, 1973; Shell, 1981). In animals, dams stressed during pregnancy can bear offspring with reduced male sexual activity, enhanced emotional reactivity (Thompson, 1957; Ward and Weisz, 1984; Weinstock *et al.*, 1988) and an increased propensity to self-administer drugs (Deminière *et al.*, 1992). Conversely, postnatal stimulation has been found to improve the performance of aged offspring in cognitive tasks (Meaney *et al.*, 1988). Although prenatal and postnatal events can have different behavioral consequences, they may also impinge on the same behavioral response, and postnatal manipulations can reverse the behavioral effects of prenatal stress. For example, postnatal handling can reverse the increase in emotional reactivity induced by prenatal stress (Wakshlak and Weinstock, 1990).

Several observations indicate that glucocorticoid secretion could be a substrate of the different long-term behavioral effects of prenatal and postnatal events. Prenatal stress increases stress-induced corticosterone secretion peak in preweaning rats (Peters, 1982; Takahashi *et al.*, 1988; Henry *et al.*, 1994) and attenuates its habituation over repeated exposure to stress in the adult (Fride *et al.*, 1986). In contrast, postnatal handling reduces stress-induced corticosterone secretion in adult and aged rats, probably by strengthening corticosterone feedback (Levine, 1962; Meaney *et al.*, 1988; Vallée *et al.*, 1996). Finally, impairment in glucocorticoid feedback, resulting in increased glucocorticoid secretion, is associated with behavioral disorders in adult (Persky, 1975; Pepper and Krieger, 1984; Holsboer, 1989) and aged individuals (Sapolsky *et al.*, 1986; McEwen *et al.*, 1986), for example, enhancing the addictive properties of drugs in animals (Piazza *et al.*, 1991).

The hypothesis of our studies, was to evaluate if a modification of fetal hormonal environment by a stress of the mother can influence the development of the activity of the hypothalamo-pituitary-adrenal (HPA) axis. Particularly, we attempted to address three main questions. First, what are the mechanisms involved in the deregulation of corticosterone secretion in prenatally stressed, adult rats? Second, what are the pathophysiological mechanisms by which stress in the mother reaches the fetus and influences its development? Third, is the HPA axis a biological substrate for the interaction between postnatal and prenatal events?

INFLUENCE OF PRENATAL ENVIRONMENT MODIFICATIONS ON THE HYPOTHALAMO-PITUITARY-ADRENAL AXIS ACTIVITY

Long-Term Consequences of a Restraint Prenatal Stress

Stress during pregnancy sensitizes different neuroendocrine systems, such as the gonads (Ward, 1972) and the HPA axis (Peters, 1982; Fride *et al.*, 1986; Takahashi *et al.*, 1988; Henry *et al.*, 1994). However, it remains unclear which mechanisms are involved in the deregulation of corticosterone secretion in prenatally-stressed adult rats. Given that hippocampal type I and type II corticosteroid receptors (Hollenberg *et al.*, 1985; Arriza *et al.*, 1987; Reul and de Kloet, 1985) appear to be major regulating factors in corticosterone secretion (McEwen *et al.*, 1986; Sapolsky *et al.*,

1986; Ratka *et al.*, 1989), we assessed stress-induced corticosterone secretion and hippocampal corticosteroid receptors in adult rats that had been submitted to prenatal manipulations (Henry *et al.*, 1994; Maccari *et al.*, 1995). Repeated restraint of the mother during the last week of pregnancy (Ward and Weisz, 1984) induces prolonged corticosterone secretion in adult offspring (90 days of age), which was indicative of impaired corticosterone feedback. Indeed, corticosterone levels in either basal conditions or 30 min after stress did not differ between the control and prenatally-stressed rats; however, corticosterone secretion was higher in the prenatally stressed than in the control rats two hours after stress (Figure 13.1a). Prenatal stress also decreased hippocampal type I corticosteroid receptors (Figure 13.1b), yet, as described by other authors (Weinstock *et al.*, 1992), prenatal stress failed to modify type II corticosteroid receptors (Figure 13.1c).

The decrease in hippocampal type I corticosteroid receptors observed in prenatally-stressed rats could account for their prolonged stress-induced corticosterone

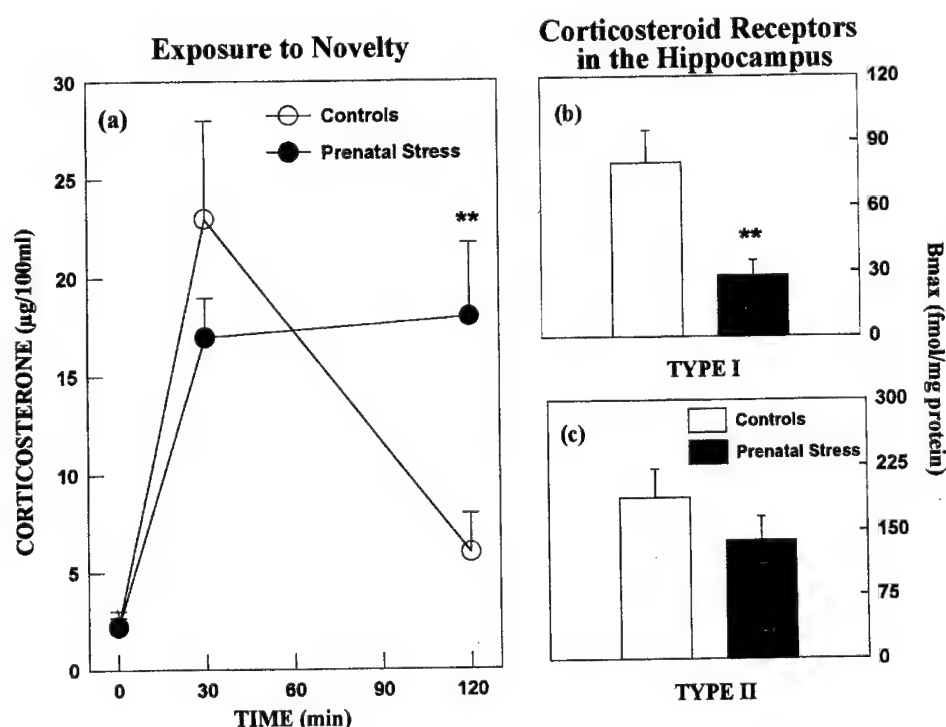


Figure 13.1 Plasma corticosterone secretion after exposure to novelty (a), type I (b) and type II (c) corticosteroid receptors in control or prenatally-stressed adult rats. (a) Prenatally-stressed animals did not differ from controls for corticosterone levels in basal conditions or after 30 min exposure to novelty. However, corticosterone levels remained high in prenatally stressed rats 120 min after stress, whereas they returned to preexposure values in controls. (b) Prenatally-stressed rats showed a lower binding capacity of type I corticosteroid receptors compared to controls. (c) Prenatal stress did not significantly modify type II corticosteroid receptors. Affinities of type I and type II receptors were not modified by prenatal stress. Mean affinities were: type I = 1.14 ± 0.11 nM, type II = 0.6 ± 0.12 nM. ** $p < 0.01$. Vertical line shows SEM.

secretion. It has been shown that a selective reduction in hippocampal corticosteroid receptors is accompanied by a prolonged corticosterone secretion in response to stress (Sapolsky *et al.*, 1986; McEwen *et al.*, 1986). In view of their affinities for corticosterone, it is generally thought that type II receptors are involved in stress-induced feedback mechanisms, while type I receptors are involved in the tonic regulation of corticosterone release under basal conditions (De Kloet and Reul, 1987). Thus, the observed decrease in hippocampal type I receptors might not be expected to be involved in stress-modulated feedback control. However, there is now evidence that both receptor types are involved in feedback control mechanisms (Ratka *et al.*, 1989; Sapolsky *et al.*, 1990; Dallman *et al.*, 1989; Maccari *et al.*, 1991). The prolonged corticosterone secretion observed in prenatally stressed animals could also account for the behavioral alterations, as for example the increased propensity to amphetamine self-administration observed in prenatally-stressed adult rats (Demiñière *et al.*, 1992). Indeed, we have previously showed that corticosterone administration can induce higher vulnerability to drugs (Piazza *et al.*, 1991).

Although mechanisms by which prenatal stress could reduce corticosterone receptors in the adult are unknown, several possibilities come to mind. For example, prenatal stress may modify glucocorticoid secretion in the adult by acting on the developing noradrenergic systems. This idea is supported by three lines of evidence. First, prenatal stress increases the turnover of brain noradrenergic neurons in adult rats (Takahashi *et al.*, 1992). Second, norepinephrine exerts a direct inhibitory control on hippocampal corticosteroid receptors and facilitates corticosterone secretion (Maccari *et al.*, 1990, 1992a). Third, norepinephrine has more influence on type I than on type II receptors (Maccari *et al.*, 1992a,b) and prenatal stress seems to affect mainly type I receptors (Maccari *et al.*, 1995). Another hypothesis could be represented by exposure *in utero* to abnormal levels of maternal corticosteroids, which cross the placental and blood-brain barriers (Zarrow *et al.*, 1970), may perturb fetal glucocorticoid receptor development. In the adult, chronic stress and repeated corticosterone administration have been found to reduce corticosteroid receptor numbers (Sapolsky *et al.*, 1984a,b; Maccari *et al.*, 1991), whereas perinatal administration of corticosteroids has been found to have neurotoxic effects in the hippocampus (Uno *et al.*, 1990).

Stress-Induced Increase in Maternal Glucocorticoids a Possible Mechanism to Explain the Long-Term Prenatal Stress Effects on the Offspring

In order to understand the pathophysiological mechanisms by which stress in the mother reaches the fetus and influences its development, we studied the effects of blocking maternal corticosterone secretion during prenatal stress on stress-induced corticosterone secretion and hippocampal corticosteroid receptors in adult offspring (Barbazanges *et al.*, 1996). Repeated restraint of the mother during the last week of pregnancy was used as prenatal stressor and dam adrenalectomy, at day 13 of pregnancy, with a corticosterone substitutive treatment (100 mg corticosterone pellet containing 50% corticosterone 21-hemisuccinate and 50% cholesterol) was used in order to block the increased in corticosterone secretion induced by the restraint stress. Furthermore, the specific role of an injection of corticosterone (3 mg/kg)

concomitantly with stress on stressed mothers adrenalectomized with corticosterone substitutive treatment was also studied on these same parameters in adult offspring. The corticosterone injections in adrenalectomized pregnant rats elicit plasma corticosterone levels approximating those found in response to stress in intact mothers.

The results show that impairment of HPA axis activity in adult offspring induced by prenatal stress depends on the high levels of maternal corticosterone secretion during restraint stress. In fact, blocking stress-induced corticosterone secretion by adrenalectomy with corticosterone substitutive treatment suppresses the prolonged stress-induced corticosterone response (Figure 13.2a) and the reduction in type I hippocampal corticosteroid receptors (Figure 13.2b) observed in prenatally-stressed rats at 3 months of age. Furthermore, administration of corticosterone to these mothers reinstates the effects of prenatal stress. Offspring of mothers in which

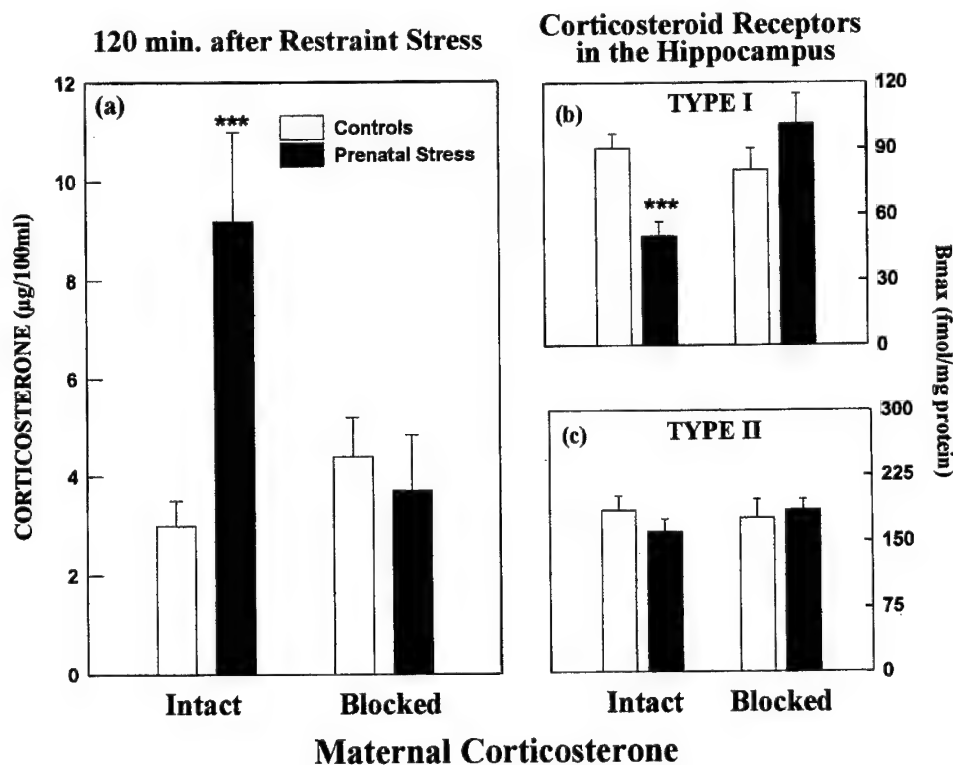


Figure 13.2 Plasma corticosterone secretion 120 min after restraint stress in control and prenatally-stressed adult offspring of mothers with intact or blocked stress-induced corticosterone secretion. (a) Prenatally-stressed adult animals whose mothers were in the intact group had higher corticosterone levels than controls, while prenatally-stressed rats whose mothers' corticosterone secretion was blocked did not differ from controls. (b) Type I corticosteroid receptors were lower in prenatally-stressed adult rats from mothers with intact corticosterone secretion, whereas prenatal stress had no effect on this measure when maternal corticosterone secretion was blocked. (c) No significant effects were found on the Bmax of type II receptors or in the affinity of either receptor type. Mean affinities (in nM) were: type I = 1.66 ± 0.17 ; type II = 1.14 ± 0.21 . *** $p < 0.001$. Error bars represent SEM.

corticosterone levels were high during stress had a longer stress-induced corticosterone secretion (Figure 13.3a) and less hippocampal type I corticosteroid receptors (Figure 13.3b) than animals whose mothers had low corticosterone levels during stress.

Taken together, these results suggest that disruption of the normal hormonal response to stress observed in prenatally-stressed individuals depends on stress-induced increase in maternal glucocorticoids. The present findings are in agreement with data showing that exposure of pregnant rats to alcohol (which stimulates maternal glucocorticoid secretion) results in a hyperactive HPA axis in the offspring (Rivier *et al.*, 1984; Lee *et al.*, 1990). Similarly, nonabortive maternal infections, which increase maternal glucocorticoids (Besedovsky *et al.*, 1975; Dunn, 1992), compromise the development of the fetal brain and alter HPA axis functioning in the adult (Reul *et al.*, 1994). However, maternal factors other than corticosterone may also contribute to long-term HPA reactivity in the adult offspring.

Of the many actions of maternal glucocorticoids during development, at least two may account for the observed effects on the offspring's HPA axis: (1) high glucocorticoid levels may alter HPA axis development by down-regulating fetal

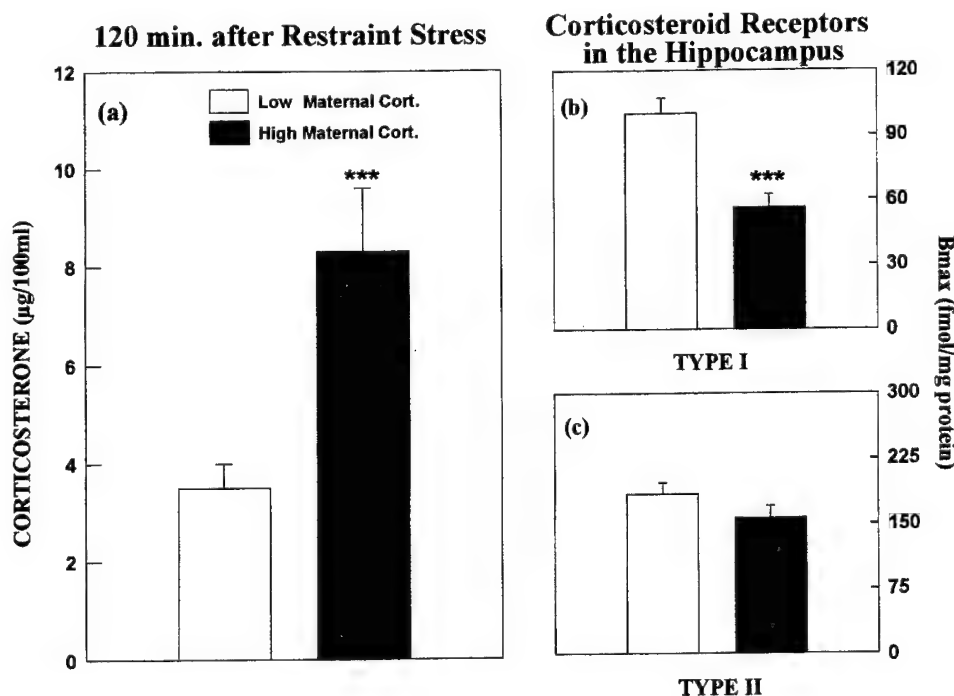


Figure 13.3 Plasma corticosterone secretion 120 min after restraint stress in prenatally-stressed adult rats whose mothers had either low or high corticosterone levels when submitted to stress during pregnancy. (a) Prenatally-stressed adult rats from mothers having high corticosterone levels had a prolonged stress-induced corticosterone secretion. (b) Type I corticosteroid receptors were lower in adult animals from mothers having high corticosterone levels. (c) No differences were found in the Bmax of type II receptor in the affinities of either receptor type. Mean affinities (in nM) were: type I = 1.71 ± 0.15 ; type II = 1.56 ± 0.30 . *** $p < 0.001$. Error bars represent SEM.

hippocampal corticosteroid receptors, which are already fully expressed during the last week of gestation (Meaney *et al.*, 1985; Rosenfeld *et al.*, 1988); (2) high glucocorticoid levels may modify glucocorticoid secretion in the offspring by acting on the developing noradrenergic systems. Indeed, prenatal stress is known to increase the turnover of brain norepinephrine in adult rats (Takahashi *et al.*, 1992) and norepinephrine exerts a direct inhibitory control on hippocampal corticosteroid receptors, thus facilitating corticosterone secretion (Maccari *et al.*, 1992a,b; Yau and Seckl, 1992).

INFLUENCE OF POSTNATAL ENVIRONMENT MODIFICATIONS ON THE HPA AXIS ACTIVITY

Influence of Postnatal Adoption on Prenatal Restraint Stress Effects

Prenatal and postnatal events affect different behaviors (Thompson, 1957; Weinstock *et al.*, 1988; Meaney *et al.*, 1988), but can also impinge differently on the same behavioral response, such that postnatal manipulations can reverse the behavioral effects of prenatal stress. For example, it has been shown that postnatal handling can reverse the increase in emotional reactivity induced by prenatal stress (Wakshlak and Weinstock, 1990). To this end, we tested if the HPA axis could be a biological substrate for the interactions between postnatal and prenatal events (Maccari *et al.*, 1995). Adoption at birth was used to perturb the postnatal environment. Pups were placed in the cage of the adoptive mother within the first 3–6 hours after birth. During this procedure, the mothers were briefly (less than 1 minute) removed from their cages. Exposure to novelty was the stress used to challenge corticosterone secretion in the adult offspring (90 days of age) and hippocampal corticosteroid receptors were also measured. We found that adoption, independently of the stress experience of the foster mother, reversed the effects of prenatal stress on both corticosterone-secretion and corticosteroid receptors. Thus, animals that were both prenatally-stressed and adopted did not differ from controls in either corticosterone secretion 2 hours after stress (Figure 13.4a) or in number of type I corticosteroid receptors (Figure 13.4b). The effects of adoption on prenatal stress were not influenced by the treatment received by the foster mother during pregnancy. Thus, adoption suppressed the effects of prenatal stress whether prenatally-stressed rats were adopted by control unstressed or stressed mothers.

Adoption *per se* had no significant effect on either corticosteroid receptor numbers or duration of the corticosterone response to stress (Figure 13.5). However, it did reduce stress-induced corticosterone secretion at 30 min after exposure to novelty. This effect of adoption was not influenced by the treatment received by the foster mother during pregnancy. Thus, stress-induced corticosterone secretion in unstressed adopted rats was lower than in controls after adoption by either a control unstressed mother or a stressed mother.

Suppression of prolonged corticosterone secretion in prenatally-stressed rats by adoption may be accounted for by its effects on type I hippocampal corticosteroid receptors. However, this mechanism cannot explain the decrease in corticosterone

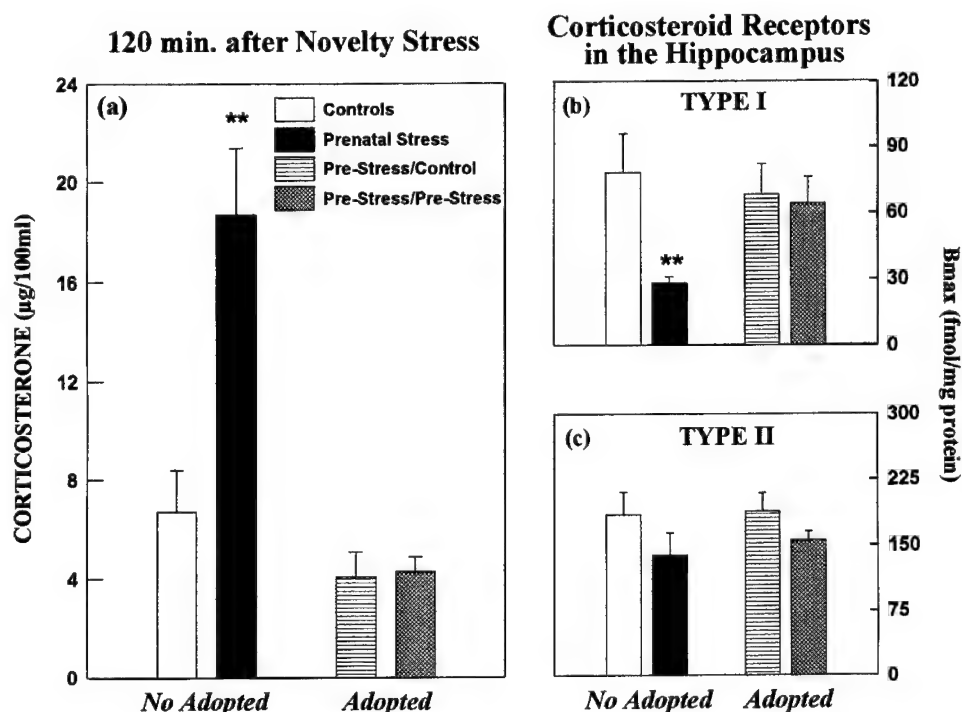


Figure 13.4 Plasma corticosterone secretion 120 min after novelty exposure (a), and type I (b) and type II (c) corticosteroid receptors in adult: Controls (prenatally unstressed rats raised by their biological mother), Prenatal stress (prenatally stressed rat raised by their biological mother), Pre-Stress/Controls (prenatally-stressed rats adopted by a control unstressed mother), Pre-Stress/Pre-Stress (prenatally-stressed rats adopted by a mother stressed during pregnancy). (a) Prenatally-stressed adult animals displayed higher corticosterone levels than those of control rats after 120 min of novelty exposure. Adult animals that were both stressed and adopted did not differ from controls, either if the adoptive mother was unstressed or stressed during pregnancy. (b) Type I corticosteroid receptors were reduced by prenatal stress and this effect was totally reversed by adoption. (c) Neither prenatal stress nor adoption significantly modified type II corticosterone receptors. The affinities of type I or type II receptors were not influenced by any of the experimental conditions studied. ** $p < 0.01$ (Prenatal Stress versus Control). Vertical line shows SEM.

secretion peak induced by adoption *per se*. This finding is not totally surprising since changes in glucocorticoid receptors, which determine the efficiency of corticosterone feedback, are more commonly associated with changes in the duration, rather than the amplitude, of corticosterone secretion (Sapolsky *et al.*, 1984b; Meaney *et al.*, 1988; Maccari *et al.*, 1991). Thus, adoption may modify corticosterone secretion via an action on the neurohormonal mechanisms involved in the secretive phase of HPA axis activity. In this respect, the effects of adoption *per se* on corticosterone secretion appear to differ from those of other postnatal stimulations. For example, postnatal handling selectively reduces the amplitude and duration of stress-induced corticosterone secretion and increases type II corticosteroid receptors (Meaney *et al.*, 1988). However, both adoption and postnatal handling converge in reducing glucocorticoid secretion, which may be a common effect of postnatal activation.

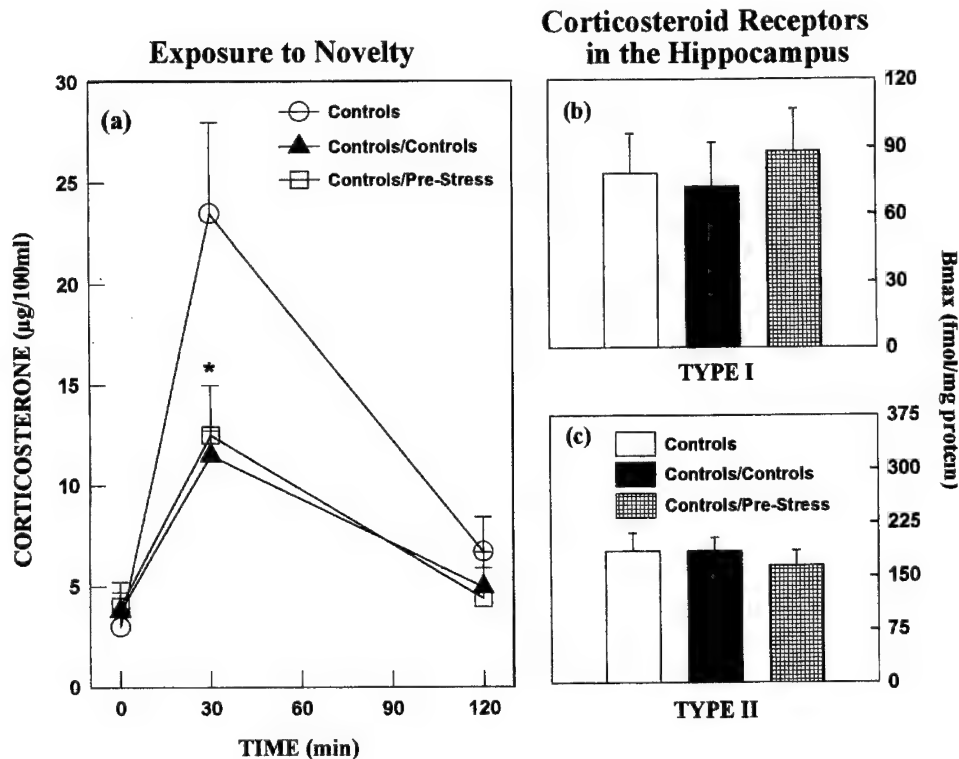


Figure 13.5 Plasma corticosterone secretion after novelty exposure (a), type I (b) and type II (c) corticosteroid receptors in adult: **Controls** (prenatally unstressed rats raised by their biological mother), **Controls/Controls** (prenatally unstressed rats adopted by a control unstressed mother), **Controls/Pre-Stress** (prenatally unstressed rats adopted by a mother stressed during pregnancy). (a) Adopted adult animals, independently of the treatment received by the mother during pregnancy, had lower corticosterone levels after 30 min of exposure to novelty than control rats. (b, c) Adoption did not significantly modify type I or type II corticosteroid receptors. The affinities of type I or type II receptors were not influenced by any of the experimental conditions studied. * $p < 0.05$ (Adopted groups versus Control). Vertical line shows SEM.

Mechanisms by which adoption in the postnatal period exercise its long-term effect on the functional state of the HPA axis remain to be elucidated. However, several hypotheses can be advanced. First, changes in maternal hormonal status may be involved in the effects of adoption *per se*. An increase in maternal corticosterone levels during the first weeks after birth (Turner and Taylor, 1976; Catalani *et al.*, 1993), which can reach the pups through the milk (Angelucci *et al.*, 1985), can induce in adult offspring a reduction in stress-induced corticosterone secretion peak comparable to that observed in adopted rats. Although seemingly contradictory, those profound differences in corticosterone's effects in the prenatal vs postnatal period might be explained by a difference in the maturational status of hippocampal corticosteroid receptors in fetus vs pup. During the last week of fetal life, levels of corticosteroid receptors are similar to those in adults, while they decrease at birth and increase again only at 12–14 days of age (Meaney *et al.*, 1985; Rosenfeld *et al.*,

1988). Second, adoption may reverse the effect of prenatal stress by a neuronal mechanism. For example, it has been shown that postnatal manipulations have a long-lasting effect on the activity of aminergic neurons (Mitchell *et al.*, 1990), which in turn can modulate the binding capacity of corticosteroid receptors (Maccari *et al.*, 1992a,b). Third, changes in maternal behavior can also play a role in the long-term functioning of the HPA axis. Indeed, an increased maternal attention and stimulation to the pups has been proposed to account for the long-term effects observed after different forms of neonatal stimulation (Bell *et al.*, 1974; Hennessy *et al.*, 1988).

Adoption-Induced Changes in Maternal Behavior as Possible Mechanism for the Effects of Postnatal Stress on the Offspring

Influence of adoption on the maternal behavior was evaluated (Maccari *et al.*, 1995) using an adoption procedure identical to that described above. Both foster and biological mothers were removed from their cages for 1 minute, and the pups were distributed around the cage. Maternal behavior was observed from the moment the mother was reintroduced into the cage. Two parameters were recorded: retrieval latency (i.e., time spent by the mother to pick up and to place each pup in the nest over 30 minutes); and time of contact (i.e., time spent by the mother licking and picking up pups over 15 minutes). These parameters provide reliable information on maternal behavior and are widely used in studies on laboratory rats (Haney *et al.*, 1989; Mann, 1993). Adoption increased maternal behavior (Figure 13.6): foster mothers spent more time licking and picking up pups (contact time) than did biological mothers. Latency to replace all the pups in the nest (retrieval latency) was also shorter with the foster than with the biological mothers. This observation is in agreement with results of another study showing that dams retrieved foster pups more quickly than their natural offspring (Misanin *et al.*, 1977).

To explain how an early adoption can have long-term effects one might first consider that modifications of mother-pup interactions are associated with a complex short-term physiologic response in the pup. For example, it has been demonstrated that activity of the HPA axis in infant rats, previously suggested to be dependent on contact with the dam (Stanton and Levine, 1990), can be regulated by experimental manipulation mimicking maternal anogenital stroking of the pups (Suchecki *et al.*, 1993). Anogenital licking of the pups has also been associated with maturation of sexual behavior in male offspring (Moore, 1984, 1992).

The fact that maternal licking behavior was increased in adoptive dams can be explained considering that maternal behavior is stimulated by olfactory and auditory stimuli from the pups. Indeed, many studies have established that maternal olfaction is involved in stimulating maternal licking (Moore and Samonte, 1986; Brouette-Lahlou *et al.*, 1991) and ultrasonic calls from the pups may induce anogenital licking of the pups by the dam (Brouette-Lahlou *et al.*, 1992). The fact that those behaviors are stimulated in the fostered dams may be due to a potential behavioral hyperresponsive period in the dams' responses to stimuli from their new pups. Furthermore, it is interesting to note that another postnatal manipulation (e.g., handling

Maternal Behavior

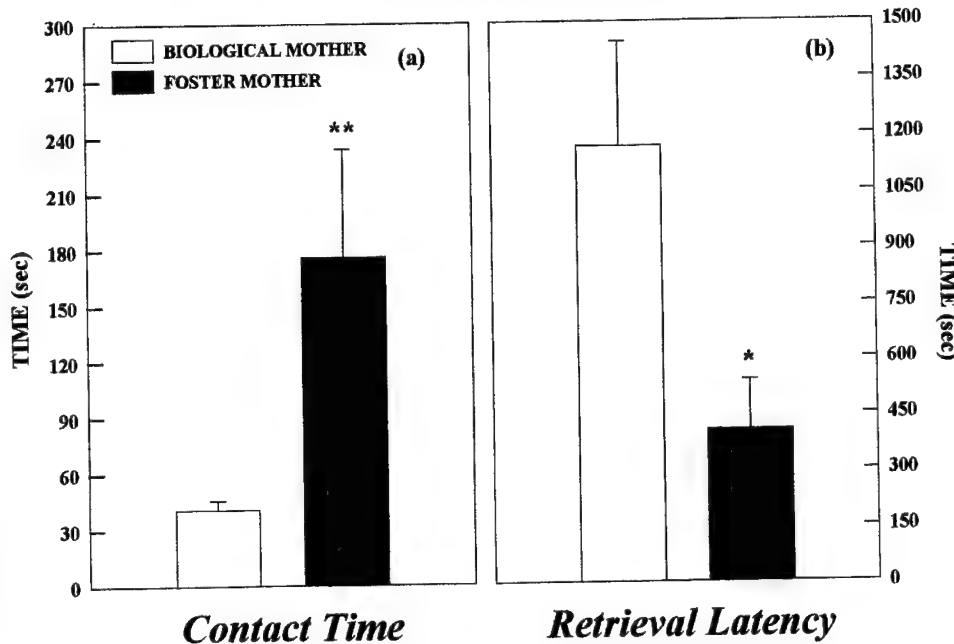


Figure 13.6 Effects of adoption on maternal behavior. (a) Foster mothers spent more time licking and picking up the pups (contact time) than did biological mothers. (b) latency to replace all the pups in the nest (retrieval latency) was shorter in foster than in adopted mothers. Duration of observation was 15 min for the contact time and 30 min for the retrieval latency. * $p < 0.05$; ** $p < 0.01$. Vertical line shows SEM.

manipulation), induces more ultrasonic vocalization in the pups (Bell *et al.*, 1971), which could increase maternal care. This may explain the behavioral and biological improvement observed in adult and aged handled rats (Meaney *et al.*, 1988).

CONCLUSIONS

In conclusion, although the development of an organism carries a strong genetic component (Bouchard *et al.*, 1990; Plomin, 1990), its early environment can have a long lasting influence. Both prenatal and postnatal events may modify the activity of the HPA axis, albeit in opposite directions, and early postnatal adoption has been found to suppress the biological effects of prenatal stress, probably by increasing maternal behavior. Our results also show the major role played by maternal glucocorticoids on the development of endocrine function in the offspring. In fact, high level of maternal glucocorticoids during prenatal stress has marked long-term repercussions on the efficiency of the offspring's HPA negative feedback mechanisms.

Finally, the recognized influence of HPA axis activity on behavioral adaptation suggests that a modification of corticosterone secretion could be a biological substrate of the long-term behavioral effects of prenatal and postnatal events.

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14 Long-Term Effects of Gestational Stress on Behaviour and Pituitary-Adrenal Function

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Stress may be defined as a challenge or threat to the organism which results in a series of reactions involving the initiation of coping strategies, defense mechanisms, somatic changes, and activation of the hypothalamic-pituitary-adrenal (HPA) and sympathetic nervous systems. These reactions help the organism adapt to the needs of the environment. However, like alarm signals, they should also be quickly turned off once the appropriate action has been taken, since a prolonged response can be detrimental. Stressful situations include those in which there is impending danger or loss of control, and the unpredictability of the outcome of one's actions, or the absence of expected reward.

Living organisms vary widely in their ability to cope in the face of psychological stress. Successful coping behavior implies the use of a learned experience that is identified with a positive outcome, such as the probability of reward and the avoidance of punishment or an adverse outcome. Persistent inescapable or unpredictable stress can lead to a failure of coping mechanisms, behavioral suppression, and dysregulation of the HPA axis, with excessive release of corticotropin-releasing hormone (CRH).

EFFECT OF GESTATIONAL STRESS ON COPING BEHAVIOUR OF OFFSPRING

Data from retrospective studies in humans and from experiments in laboratory animals suggest that coping behavior under stress and the appropriate response of the HPA axis can both be strongly influenced by events that occurred during the fetal and neonatal period. Thus, different forms of psychological stress experienced by the pregnant mother, such as marital and family discord (Stott, 1973), the threat of impending war (Meier, 1986), or death of the spouse (Huttunen and Niskanen, 1978), produced behavioral abnormalities in their children, consistent with impaired ability to cope with situational demands. These included excessive crying and clinging

to the mother, low frustration threshold, and unsociable and inconsiderate behavior towards other children. There appeared to be a greater incidence of juvenile crime, depressive and neurotic disorders and alcohol intake in prenatally-stressed (PS) teenagers (Huttunen and Niskanen, 1978), but follow-up studies into adulthood are lacking.

Because of the difficulty of relating gestational events to behavior in human subjects most of our information on the influence of prenatal stress on coping behavior of the adult offspring and on regulation of their HPA axis is derived from studies in experimental animals. Pregnant rodents or monkeys have been subjected to a variety of stressful situations, including unavoidable electric shocks, restraint, overcrowding, chronic social stress, or unpredictable noise. The effect on the offspring is much more marked if the dam cannot adapt to the stress or control it (Weinstock *et al.*, 1988). While prenatally-stressed (PS) offspring do not differ from controls in food seeking (Fride *et al.*, 1986), maternal behavior (Fride *et al.*, 1985), or exploration in familiar surroundings (Poltyrev *et al.*, 1996) under normal conditions, their performance in these and other tasks becomes significantly more disrupted when the situation is aversive, or is conflict inducing. Examples of such situations include mild electric footshocks (Fride *et al.*, 1986; Takahashi *et al.*, 1992a), cold air puffs (Fride *et al.*, 1985), and removal to a noisy, novel environment (Clarke and Schneider, 1993) (Table 14.1). PS rats also show a greater behavioral suppression than controls in the forced swimming test (Alonso *et al.*, 1991) and in the elevated plus maze (Fride and Weinstock, 1988). The latter situation is purported to measure anxiety, as it presents a conflict between the rat's exploratory behavior and its putative "fear" of heights and open spaces.

The behavioral suppression that is induced in normal adults by subjecting them to repeated uncontrollable stress is associated with raised amounts of circulating corticosterone (CORT), and can be prevented by pretreatment with metyrapone,

Table 14.1 Response latencies to various stimuli and suppression of behavior by aversive conditions in control and PS offspring

Behavior	Controls		Prenatal Stress	
	Normal	Aversive	Normal	Aversive
Pup Retrieval (female rats) ¹				
Time to contact with pups (sec)	87±24	219±28	52±12	327±67†
Time to retrieve all pups (sec)	243±33	341±38	175±26	407±75†
Pups retrieved (%)	100	96	100	52†
Food seeking (female rats) ² latency (sec)	14.2±1.4	24.4±4.6	9.9±1.2*	125±10†
Exploration (male and female monkeys) ³ time spent (sec)	22.5±3.5	7.5±4	31±1.4	2±1†
Freezing (male rats) duration (sec) ⁴	—	407±29	—	489±18*
Immobility (female rats) % of test time ⁵	—	50±4	—	67±4*

†Significantly greater change from normal conditions than in controls, $p < 0.05$; *significantly different from value in controls, $p < 0.05$.

¹Fride *et al.* (1986). *Physiol. Behav.*, **37**:681–687.

²Fride *et al.* (1985). *Life Sci.*, **36**:2103–2109.

³Clarke and Schneider (1993). *Dev. Psychobiol.*, **26**:293–304.

⁴Takahashi *et al.* (1992). *Brain Res.*, **574**:131–137.

⁵Alonso *et al.* (1991). *Physiol. Behav.*, **50**:511–517.

which interferes with CORT synthesis (Baez and Volosin, 1994). Chronic, uncontrollable stress augments the release and action of CRH by disrupting the normal feedback regulation of the HPA axis mediated by glucocorticoids through specific receptors in the hippocampus and other brain regions (Jacobsen and Sapolsky, 1991). In addition to promoting the release of ACTH and β -endorphin from the anterior pituitary gland via the portal circulation, CRH stimulates noradrenaline (NA) cell bodies in the locus coeruleus. This increases NA turnover in the paraventricular nucleus (PVN), thereby augmenting the release of CRH, as part of a positive feedback loop (Chrousos, 1992). Injection of CRH into the brains of naive rats induces behavior consistent with that of hyperanxiety in novel situations, and includes suppression of locomotion and social interaction, avoidance of the open arms of the plus maze, and immobility in the forced swimming test (Dunn and Berridge, 1990).

EFFECT OF GESTATIONAL STRESS ON REGULATION OF THE HPA AXIS

The similarity between the behavioral effects of CRH administration and those seen in PS animals suggests that gestational stress may sensitize the fetal HPA axis to the influence of life stresses and induce permanent alterations in its feedback regulation. The greater amounts of CRH, ACTH and CORT released in response to stressful situations could induce more behavioral suppression than that seen in control subjects. This hypothesis has been confirmed in different studies on the regulation of the HPA axis after gestational stress, which found that plasma ACTH and CORT were significantly higher in 14-day-old male PS than in control rat pups under resting conditions. This difference disappeared at 21 days of age (Takahashi and Kalin, 1991), but was still present in adult PS females (Weinstock *et al.*, 1992). In response to a variety of stressful situations (e.g., unfamiliar environments, electric footshocks, noise or overcrowding) PS rats or monkeys of either sex release greater amounts of pituitary and adrenal hormones (Clarke *et al.*, 1994; Fride *et al.*, 1986; Henry *et al.*, 1994; Weinstock *et al.*, 1992). Plasma CORT does not only rise to higher levels at the time of peak response to stress in PS rats but declines more slowly to baseline values than in controls (Weinstock *et al.*, 1992; Maccari *et al.*, 1995). The greater reactivity of the HPA axis in PS rats of both sexes to different manipulations performed in our laboratory is shown in Figure 14.1. While amounts of circulating CORT did not differ at rest in naive control and PS rats aged 7 weeks, they increased much more in the latter in response to relatively mild procedures such as saline injection, or placement in the open field for 5 minutes.

Evidence of sensitization of the HPA axis of PS rats by prior experience is demonstrated by the finding that exposure to an unfamiliar environment resulted in a significant elevation of basal CORT levels, measured 10–20 days later. Unlike controls, PS rats also fail to adapt to repeated subjection to a stressful environment, as shown by the fact that they voided more fecal pellets, explored less, and released significantly higher amounts of CORT throughout the eight consecutive exposures to the same open field (Fride *et al.*, 1986).

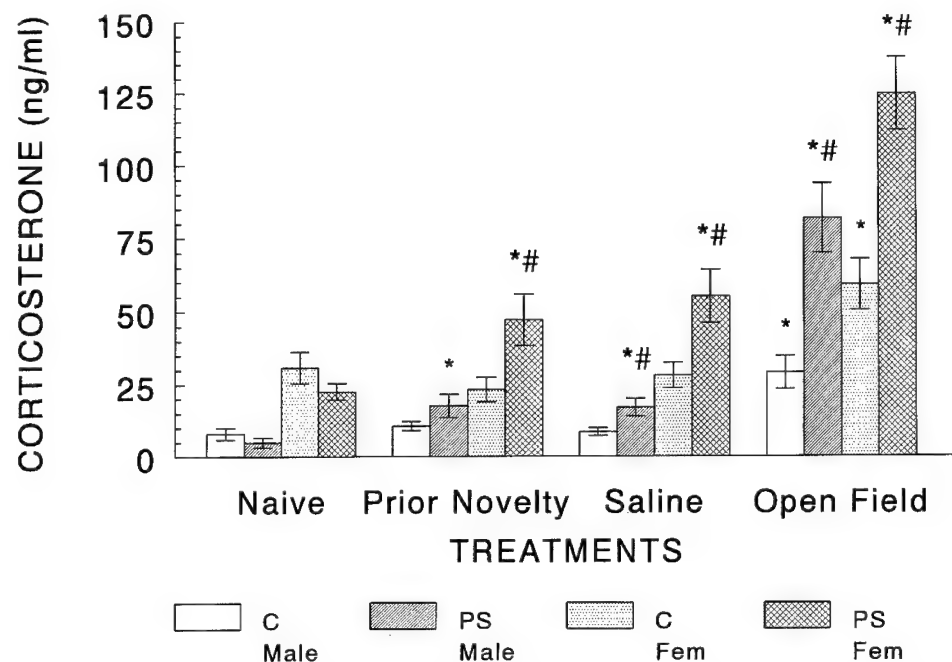


Figure 14.1 The effect of prenatal stress on plasma CORT levels in naive rats of both sexes and after exposure to different stimuli. Measurements were performed in rats housed 2 days previously in individual cages. Blood was taken 30 min after saline injection or exposure to the open field. *Significantly different from naive animals, $p < 0.05$; #significantly different from controls, $p < 0.05$.

CORT inhibits the activation of the HPA axis by binding to two types of specific cytosolic receptors in the hippocampus and other sites, with different affinities for the steroid. Weinstock *et al.* (1992) showed that intact PS female rats had a lower number of glucocorticoid binding sites in the hippocampus under resting conditions and after exposure to a novelty stress. The apparently selective effect of prenatal stress in females in this study may have been due to their two- to threefold higher circulating levels of CORT than in males. By using specific ligands for the two subtypes of glucocorticoid receptors, Henry *et al.* (1994) found that both types I and II were reduced in adult PS males (females were not tested) but in a later study, this group only obtained a significant effect of prenatal stress on type I receptors (Maccari *et al.*, 1995). This issue remains to be clarified.

Additional evidence in support of an alteration in the activity of the HPA axis by prenatal stress includes the finding of lower amounts of proopiomelanocortin in the hypothalamus of prepubertal female rats (Weinstock *et al.*, 1992) and a higher turnover of NA in the cortex and locus coeruleus in PS males at rest and after exposure to footshock (Takahashi *et al.*, 1992b). Although we did not find any significant differences in the levels of CRH or vasopressin in the median eminence of naive PS and control rats (Tilders and Weinstock, unpublished observations), PS rats do appear to have higher amounts of CRH in the amygdala (Cratty *et al.*,

1995). Furthermore, stimulation by KCl of amygdala minces from these rats releases significantly greater amounts of the peptide than from those of controls. Activation of CRH receptors in the amygdala induces anxiety that may be overcome by stimulation of benzodiazepine receptors in the hippocampus or the GABAergic system in the amygdala (Davis *et al.*, 1994). The finding that additional CRH can be released from the amygdala, combined with the presence of fewer benzodiazepine binding sites in the hippocampus in PS rats (Fride *et al.*, 1985), could explain the greater degree of fear and behavioral suppression exhibited by PS animals in aversive or unfamiliar situations.

PS rats have a higher rate of dopamine (DA) turnover in the prefrontal cortex (Fride and Weinstock, 1988) and a lower rate than controls in the nucleus accumbens and striatum (Fride and Weinstock, 1988; Alonso *et al.*, 1994). This is consistent with their impaired coping behavior and hyperanxiety in unfamiliar situations, since similar changes can be induced by the administration of the anxiogenic agent, β -carboline (Tam and Roth, 1985). The greater behavioral suppression of PS rats in stressful environments is consistent with a decrease in DA release in the nucleus accumbens (Alonso *et al.*, 1994).

HOW DOES GESTATIONAL STRESS MEDIATE THE ALTERATIONS IN COPING BEHAVIOUR AND REGULATION OF THE HPA AXIS?

Since there are no direct neural connections between the developing fetus and the mother, maternal stress must induce the changes in the function of the fetal endocrine system by hormones which reach it via the placental circulation. Attempts have been made to identify the hormones responsible. Restraint during late pregnancy raised plasma CORT in rat mothers and their fetuses, but other hormones were not measured in this study (Ohkawa *et al.*, 1991). In order to mimic the effects of prenatal stress, CORT was injected to the rat dams on days 19 or 20 of gestation in doses that raised plasma levels to those obtained after noise stress. As found in PS rats, early motor development was slowed and the time spent in the open arms of the plus maze in adulthood was decreased (Gavish and Weinstock, unpublished observations). Daily injection of ACTH for two weeks from day 120 of gestation in rhesus monkeys, which also elevated maternal CORT, delayed development of early motor coordination and decreased attention span in the infants, in a manner similar to that seen after psychological maternal stress during midgestation (Schneider *et al.*, 1992). These findings suggest that some of the behavioral sequelae of prenatal stress may be mediated by the action of excess CORT on the developing fetal brain.

Other hormones such as β -endorphin released in the dam (Falconer *et al.*, 1988) and from the fetal hypothalamus and pituitary during stress at late gestation (Rohde *et al.*, 1989) may also influence development and behavior of the offspring. Thus, the feminization of male sexual behavior and increased timidity in novel surroundings induced in rats by gestational stress can be replicated by maternal administration of opioid drugs (Ward, 1983; Vathy *et al.*, 1985). On the other hand, the effects of prenatal stress on male sexual behavior (Ward *et al.*, 1986), the delays in early

development, and hyperanxiety in the plus maze (Keshet and Weinstock, 1995) can be prevented by continuous administration of the opioid antagonist naltrexone to the stressed mother during the last week of gestation. It is not clear how excess opioid activity during fetal development alters behavior in the adult, but a reduction in the number of opioid receptors (Insel *et al.*, 1990) and of saccharin preference, an opioid dependent behavior (Keshet and Weinstock, 1995), was found in adult PS rats.

CONCLUSIONS

Exposure to psychological stress during gestation can induce long-lasting behavioral changes in the offspring, characterized by a decreased ability to cope with, or adapt to, stressful situations. This is associated with dysregulation of the HPA axis, resulting in elevated blood levels of ACTH and CORT in response to stress, which do not lessen on repeated exposure. It is possible that these changes are due to excess activity of CRH because of a deficient feedback control of its release or action. The alterations in behavior and HPA axis regulation appear to result from abnormal hormonal activity in both the mother and fetus; these modifications can affect the developing fetal brain at a critical time during development.

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IV. Neuroimmunology of the Stress Response

15 Steroids, Stress and the Neuroimmune Axis

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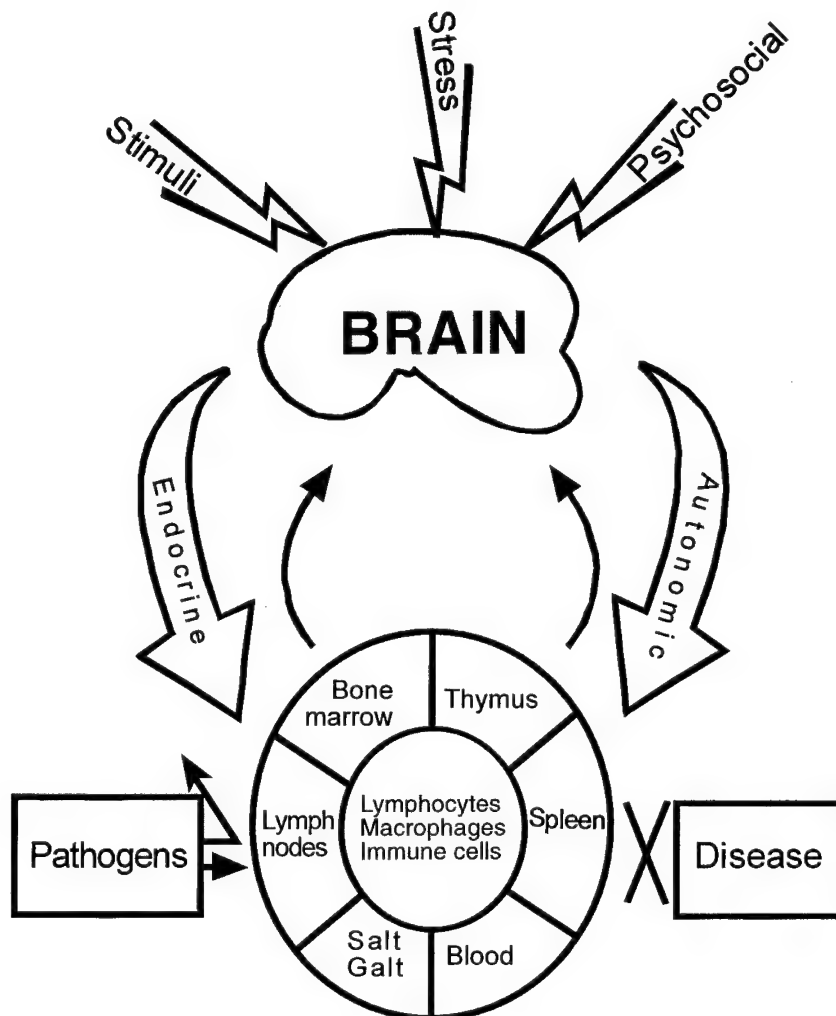
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The existence of an interaction between the nervous, endocrine and immune systems has been established. Physical, social, or pharmacologically-induced stress, as well as infections result in elevation of ACTH-mediated adrenal glucocorticosteroids levels. Such increases of hydrocortisone in humans and corticosterone in mice induces immune suppression resulting in involvement of the neuroimmune endocrine axis. In contrast, dehydroepiandrosterone (DHEA) and two of its metabolites, androstenediol (5-androstene-3 β -17 β -diol, AED) and androstetriol (5-androstene-3 β -7 β -17 β -triol, AET) upregulate host immune response against infections and counteracts stress-induced immune suppression *in vivo*. Indeed, DHEA, and particularly AED and AET, have been shown to protect mice from viral, bacterial, and parasitic infections. This protective activity has been attributed, in part, to their ability to counterbalance the immune-suppressive effects of glucocorticoids *in vivo*. The penetration of the noninvasive, attenuated encephalitis viruses, West Nile and Sindbis, into the brain and their replication is enabled by cold, isolation, or pharmacological stress. Injection of DHEA to these infected animals protects the host from this stress-mediated, virus-induced mortality. In order to determine the individual activity of each of these steroids the *in vitro* influences of DHEA, AED, and AET on a mitogen-induced mixed splenocyte proliferation assays were determined. The results showed that DHEA suppressed the proliferation of concanavalin A- (ConA-) or lipopolysaccharide- (LPS) activated cultures in a dose dependent manner. AED had little influence on the activation response. However, AET potentiated the response to both mitogens significantly above control. The regulation of the cytokines interleukin-2 (IL-2) and interleukin-3 (IL-3) secretion from ConA-activated lymphocytes was affected in the same manner. These functions were depressed by DHEA, unaffected by AED, and potently increased by AET. Moreover, the classic immunosuppressive effects of hydrocortisone on ConA-induced lymphocyte proliferation, as well as IL-2 and IL-3 production were unaffected by coculture with DHEA and only minimally counteracted by AED. In contrast, AET significantly counteracted the effect of hydrocortisone when cocultured. These data show that while *in vivo*, DHEA, AED, and AET may have some similar functions, their effects are dramatically different from one another *in vitro*. Only AET could markedly potentiate the cellular response by increasing lymphocyte activation and counteracting the immune-suppressive activity of hydrocortisone.

In summary, the *in vivo* effects associated with DHEA, AED and AET suggest that their antistress effects may be mediated in part by their function in opposition of glucocorticoids, and by upregulating the host immune responses.

The existence of a connection and interaction between the nervous and immune systems has been established (Besedovsky and Sorkin, 1977; Smith and Cuzner, 1994; Spanello and Gorospe, 1995). The major pathways of interactions between the CNS, the endocrine and the immune systems in health and disease, are illustrated in Figure 15.1. Our previous observations have shown the importance of host factors on



Adapted from: Cohen, A., Ader, R., and Felten, D.L. Psychoneuroimmunology, in Sigal, L.H. and Ron, Y. eds. "Immunology and Inflammation: Basic Mechanisms and Clinical Consequences". McGraw Hill Inc. 1994 (with permission).

Figure 15.1 The interactions between the CNS, the endocrine and the immune system in health and disease is illustrated.

resistance and susceptibility to infections (Loria, 1988). Since then, we expanded our investigation into the role of steroids hormones in neuroimmune regulation.

The sulfated and water soluble form of dehydroepiandrosterone (5-androstene-3 β -ol-17-one, DHEA) is quantitatively the major secretory product of the human adrenal gland (Tyrell and Forsham, 1983). Aging markedly decreased the level of DHEA, reaching about 5% of the original level in the elderly (Carlstrom *et al.*, 1988). The biological activities of DHEA have been described and reportedly include: (a) reducing body weight by regulating food efficiency ratio (Yen *et al.*, 1977; Schwartz *et al.*, 1981); (b) inhibiting of mammalian glucose 6 phosphate dehydrogenase (G6PD) (Tsutsui *et al.*, 1962; Pashko *et al.*, 1981); (c) inhibiting of DNA and RNA synthesis and an antitumor effect (Schwartz *et al.*, 1981); (d) An antidiabetic effect and reduced blood glucose tolerance (Coleman *et al.*, 1982); (e) inhibiting lipid synthesis (Ben-David *et al.*, 1967; Kritchevsky *et al.*, 1983; Nestler *et al.*, 1987); and (f) functioning as an antagonist of the GABA_A receptor (Majewska *et al.*, 1990).

DEHYDROEPIANDROSTERONE (DHEA) AS AN IMMUNE UP-REGULATOR

Two acute virus infection models in the C57BL/6J inbred mouse with distinctive replicative and pathogenic mechanisms were examined for their response to DHEA following infection. The results showed a reduced mortality of 50% following a challenge of male mice with a lethal dose of *Coxsackievirus B4* (CB4), female mice with *Herpesvirus type 2* (HSV2), or *Enterococcus faecalis*, when injected with a single subcutaneous (sc) injection of DHEA, (Loria, 1988, 1990). The findings showed that DHEA treatment reduced mortality in animals infected with CB4 at LD₁₀₀ dose (10⁵ pfu/animal) from 90% to 37.5%. HSV2-induced mortality was reduced from 88% to 0% at an infectious dose of 10⁷ pfu/animal. This protective effect of DHEA against intraperitoneal (ip) CB4 or intracranial HSV2 infections was statistically significant, $p < 0.03$. Similarly, a single subcutaneous injection of DHEA protected mice from infection by a lethal dose of gram positive bacteria *Enterococcus faecalis*, and mortality was reduced from 95% to 60% (Loria *et al.*, 1988a, 1990). Since DHEA had no direct effect on either viral or bacterial replication *in vitro*, and failed to function in the genetically immune impaired mutants HRS/J *hr/hr* mice, we surmised that its protective effect is derived from stimulation of the immune response (Loria *et al.*, 1988a). Moreover, it was unlikely that this hormone would have a direct antiviral or antibacterial action on such a wide range of different organisms (Table 15.1).

In order to determine the effect of DHEA on the immune response, we examined the number of spleen IgM antibody forming cells (AFC) in sheep red blood cell-immunized, CB4-infected and uninfected mice. The numbers of AFC per 10⁶ spleen cells from CB4-infected DHEA-treated mice were 80% higher than the number of IgM AFC in CB4-infected control mice, $p < 0.025$. In comparison, uninfected DHEA-treated mice had only a 35% increase in the numbers of AFC; this increase was not statistically significant compared to the uninfected control mice. The number of spleen IgG AFC were also enumerated. A 70% increase observed in DHEA-treated and

Table 15.1 *In vivo* DHEA's range of protective effects

<i>Agent</i>	<i>Class</i>	<i>Family</i>	<i>Strain</i>
Viruses	RNA	Picornavirus	Coxsackievirus B4 ¹
		Flavivirus	West Nile Virus ²
		Alphavirus	Semliki Forest Virus ²
		Retrovirus	Murine mammary tumor virus ³
	DNA	Herpesvirus	Herpes Type 2 ¹
Bacteria		Gram Positive	Enterococcus faecalis ¹
		Gram Negative	Pseudomonas aeruginosa ²
Parasites		Coccidia	Cryptosporidium parvum ⁴
Non infectious		Lipopolysaccharide	7,12 dimethyl benz (A) anthracene and urethane induced tumors ^{3,5}

¹ Loria, 1990; ² Ben-Nathan *et al.*, 1991; ³ Schwartz, 1979; Schwartz *et al.*, 1981; ⁴ Rasmussen *et al.*, 1992; ⁵ Li *et al.*, 1994.

CB4-infected mice as compared to virus-infected mice not treated with DHEA was not statistically significant. The range of DHEA protection against various forms of infection and tumors is summarized in Table 15.1.

We have found that the route of DHEA administration is important since delivery as a single dose via ip or intravenous (iv) routes did not protect against lethal infectious challenge. When provided as a dietary supplement, DHEA protected the host against infection. However, protection increased dramatically when delivered by the subcutaneous route. This suggests that the cutaneous route of delivery is optimal for immune upregulation for antiviral and antibacterial results. Indeed, DHEA is converted in the skin to androstenediol (5-androstene-3 β ,17 β -diol, AED) and androstenetriol (5-androstene-3 β ,7 β , 17 β -triol, AET). This metabolic pathway appears to predominately reside within the skin and brain (Berliner and Gallegos, 1967; Berliner *et al.*, 1968; Faredin *et al.*, 1969; Toth and Faredin, 1983; Vourc'h *et al.*, 1992; Akwa *et al.*, 1992; Mathur *et al.*, 1993). Consequently it was postulated that the cutaneous metabolic conversion of DHEA could result in the mobilization of the skin-associated lymphoid tissue (SALT) and could account for the observed immune mediated protective activity. In order to test this hypothesis the effects of AED and DHEA on lethal CB4 infections in C57BL/6J mice were compared (Loria and Padgett, 1992a).

The results show that CB4 infection with 10⁴ pfu/animal was lethal to all six mice tested, whereas five out of six mice survived a single subcutaneous injection of DHEA (1.0 g/kg). With AED, a lower dose of 320 mg/kg was sufficient to confer protection to all six mice infected with the same virus dose. At a higher virus dose of 10⁶ pfu/animal CB4, equivalent to 100 times the LD₁₀₀ for the C57BL/6J strain, DHEA offered no protection, while AED at 1/3 the dose conferred protection to six out of six infected animals. Thus, AED, a metabolite derived from DHEA, is markedly more efficient in providing immune upregulation than its precursor (Loria and Padgett, 1992b).

Since genetic factors play a role in the outcome of enterovirus infection, the dose effect of AED in SWR/J and C57BL/6J inbred mice were compared. At AED doses of 20, 80, 160, or 320 mg/kg body weight, AED effectively protected SWR/J mice

from a lethal virus infection. Androstenediol at a concentration of 10 mg/kg body weight, provided a theoretical effective dose (extrapolated) resulting in 50% survival (ED_{50}) from an infection with 10^8 pfu/animal of human coxsackievirus B4. In the SWR/J(q) inbred mouse, AED at a dose of 10 mg/kg is 100 times more potent than DHEA, which was protective at a dose of 1.0 g/kg. In the C57BL/6J(b) inbred mouse, a higher dose of AED (70 mg/kg) was required to achieve an ED_{50} . The data showed that androstenediol (AED) is significantly more effective in protecting the host against viral mediated mortality with AED being approximately 10,000 times more efficacious than DHEA.

Similar comparisons of the effects of DHEA and AED on bacterial infections were carried out. Previously we reported a 50% reduced mortality when treated with either DHEA and AED following challenge by a lethal dose of *Enterococcus faecalis* (Loria *et al.*, 1990). Presently, we also report a significant protective effects against *Pseudomonas aeruginosa*. DHEA protected 50% of mice challenged with 10^7 CFU/mouse of *P. aeruginosa*, while AED protected more than 70% of similarly challenged animals.

In vivo, particularly in the skin and the brain, DHEA is metabolized along the pathway DHEA \rightarrow AED \rightarrow AET (Berliner and Gallegos, 1967; Berliner *et al.*, 1968; Faredin *et al.*, 1969; Toth and Faredin, 1983; Vourc'h *et al.*, 1992; Akwa *et al.*, 1992; Mathur *et al.*, 1993). In order to examine the possible role of AET, this hormone was synthesized and tested for its effects on host resistance against a lethal infection with coxsackievirus B4. The results show that AET is more potent than AED, and that the order of immune upregulation obtained with these steroids is DHEA \lll AED $<$ AET. Since all three agents had activity *in vivo*, it was important to determine their independent activities as well as which of these steroids was actually the most effective as an immune regulator. To achieve this goal, the specific effects of each agent on mitogen-stimulated activation of murine lymphocytes were measured *in vitro* (Padgett and Loria, 1994).

IN VITRO STUDIES COMPRISING THE EFFECT OF DHEA, AED, AND AET

The influence of DHEA and its metabolites, AED and AET, on the activation of the immune system was examined. The activation phase is the sequence of events induced in lymphocytes leading to expansion of antigen-specific clones. It is generally assumed that polyclonal activators mimic antigens. Therefore, the changes induced are similar to those induced by specific antigens on their respective antigen-specific clone. DHEA inhibited the uptake of [3 H] thymidine by ConA-induced C57BL/6J splenocytes in a dose dependent manner. The proliferative response of splenocytes treated with doses of DHEA ranging from 5.0×10^{-6} M to 5.0×10^{-9} M was typically decreased 25–40% from control ConA-stimulated cultures. The influence of AED on ConA-activated C57BL/6J splenocytes was minimal. Splenocytes treated with AED at doses ranging from 5.0×10^{-6} M to 5.0×10^{-9} M had [3 H]thymidine incorporation rates ranging from 95–105% of control splenocytes treated with 2.5 g/ml ConA. AET independently increased the proliferation of ConA-stimulated

C57BL/6J splenocytes. At each dose ranging from 5.0×10^{-6} M to 5.0×10^{-9} M, AET increased [3 H]thymidine incorporation from 50% to 70% above control, in sharp contrast to the suppression by DHEA. Such an observation supports our hypothesis that conversion from DHEA to AED and finally to AET is important for the beneficial immunomodulatory effects attributed to each of these steroid hormones *in vivo*. This data suggest that while DHEA and AED did not stimulate lymphocyte proliferation *in vitro*, *in vivo* delivery results in at least a partial conversion to AET that is potentially capable of supporting the proliferative response of the same cells. Similar results were obtained when LPS was used as the mitogen.

These data show that, regardless of the stimuli, these steroid hormones possess similar activity with AET which enhanced significantly the lymphoproliferative action of mitogens.

EFFECTS OF DHEA, AED, AND AET ON INTERLEUKIN-2 AND INTERLEUKIN-3 PRODUCTION

The activation and coordination of lymphocytes are dependent upon the production of cytokines. These molecules act on lymphocytes and on many other cell types including macrophages. The ability of steroid hormones to regulate T-cell function and cytokine production is a direct evidence of their role as regulatory molecules governing the immune system. Therefore, in order to gain a better understanding of their impact on the immune system, we examined the influence of DHEA/AED/AET on the secretion of specific cytokines, IL-2 and IL-3. IL-2 is a critical factor in maintenance of immune homeostasis. The effects of each of these steroid hormones on the production of IL-2 by ConA-stimulated C57BL/6J murine T-lymphocytes. Concentrations of DHEA ranging from 5.0×10^{-6} M to 5.0×10^{-9} M inhibited the production of IL-2 by 18–23% as compared to control Con-A stimulated cultures. The influence of AED on ConA-stimulated IL-2 production was minimal. AED doses ranging from 5.0×10^{-6} M to 5.0×10^{-9} M produced IL-2 at 97–107% of control. While AET doses from 5.0×10^{-6} M to 5.0×10^{-9} M AET increased IL-2 production by 111–122% above control. The effects of each of these steroid hormones on the production of IL-3 by ConA-stimulated C57BL/6J murine T-lymphocytes were also measured. Concentrations of DHEA ranging from 5.0×10^{-6} M to 5.0×10^{-9} M inhibited the production of IL-3 by 20–48% as compared to control ConA-stimulated cultures. The influence of AED on ConA-stimulated IL-3 production was minimal. AED at doses ranging from 5.0×10^{-6} M to 5.0×10^{-9} M produced IL-3 at 97–108% of control cultures. In contrast, AET ranging from 5.0×10^{-6} M to 5.0×10^{-9} M AET increased IL-3 production by 30–47% above control. Clearly, DHEA and AED do not stimulate IL-3 production *in vitro*. The results on IL-2 and IL-3 suggest that the activation and proliferation of murine lymphocytes are partially mediated by the production of T-cell growth factors such as IL-2 and IL-3. AET is more effective in enhancing the production of these cytokines and augmenting lymphocyte activation and proliferation. Moreover, because DHEA and AED failed to enhance cytokine secretion, the data implies that *in vitro* murine splenocytes do not convert DHEA to AED, nor AED to AET.

STRESS MEDIATED MORBIDITY AND MORTALITY

Ben-Nathan *et al.* (1991, 1992a,b) reported that stress mediated immunosuppression, induced by social isolation, physical exposure to cold, or glucocorticoid-induced stress (dexamethasone) causes a marked increase in mortality from an attenuated West Nile virus or Simliki Forest virus. This stress-mediated increase in mortality could be offset by treatment with DHEA. Moreover, it was apparent that stress from either isolation or cold is mediated by a marked and significant increase in the replication of an attenuated strain of West Nile encephalitis both in the spleen and the brain. Moreover, this stress-mediated increase in virus replication in the brain was directly associated with increased mortality.

Based on the results presented it was proposed that DHEA, AED and AET would function by controlling the host immune response to maintain homeostasis, since many of the *in vivo* observations contrast directly with the established effects attributed to glucocorticoids. Consequently, we tested the effects of each of these steroids *in vitro* on their ability to counteract glucocorticosteroid effects.

EFFECTS OF DHEA, AED AND, AET ON GLUCOCORTICOID-INDUCED SUPPRESSION OF SPLENOCYTE PROLIFERATION

Glucocorticoids have been used extensively to inhibit inflammation. The antiinflammatory process is mediated in part by interfering with the activation and function of lymphocytes (Boumpas *et al.*, 1991; Scudeletti *et al.*, 1990). Hydrocortisone at doses ranging from 5.0×10^{-6} M to 5.0×10^{-8} M inhibited ConA-induced proliferation of splenocytes from C57BL/6J mice in a dose-dependent manner. To further investigate the function of DHEA, AED, and AET, the antiglucocorticoid activities of these hormones were assessed by coculturing them with hydrocortisone-treated murine splenocytes. DHEA at doses from 5.0×10^{-6} M to 5.0×10^{-8} M did not counteract the effects of hydrocortisone. AED partially blocked the hydrocortisone-mediated suppression at higher doses of 5.0×10^{-6} M. In contrast, AET completely inhibited the suppression mediated by 1.0×10^{-7} M hydrocortisone. In fact, not only did AET block the antiproliferative effects of hydrocortisone, but it stimulated the activation and proliferation of the splenocytes from 105% to 127% above control levels. These observations support the hypothesis that conversion from DHEA to AED and subsequently to AET is important for the observed *in vivo* antiglucocorticoid activity associated with each of these steroid hormones.

EFFECTS OF DHEA, AED, AND AET ON GLUCOCORTICOID-INDUCED SUPPRESSION OF IL-2 AND IL-3 SECRETION

Hydrocortisone at doses ranging from 5.0×10^{-6} M to 5.0×10^{-8} M inhibited ConA-induced IL-2 and IL-3 secretion by splenocytes from C57BL/6J mice in a dose-dependent manner, resulting in 77% and 49% inhibition, respectively. To further investigate the function of DHEA, AED, and AET, the antiglucocorticoid activities

of these hormones were assessed by coculturing them with hydrocortisone-treated murine splenocytes. DHEA did not negate the effects of hydrocortisone at any of the doses tested. AED offered marginal counteracting capacity; it completely inhibited the suppression mediated by 1.0×10^{-7} M hydrocortisone and blocked the effects of hydrocortisone and stimulated secretion of IL-3 significantly from 98.4% to 114% above control levels, respectively. The results also show similar effects with regard to the production of IL-2 from the same ConA-stimulated T lymphocytes. Again, as with activation and proliferation of murine splenocytes, AET possessed strong antiglucocorticoid activity in terms of cytokine production. Interestingly, these results, coupled with lymphocyte activation, suggest that at least in this domain, the functions of DHEA are similar to glucocorticosteroids.

These findings warrant the conclusion that AET partly mediates an increase in the T_H1 response. This conclusion is supported by the overall cytokine pattern, namely IL-2 and IL-3 production obtained with AED and AET. This is in contrast to glucocorticosteroids, which stimulated IL-4 and suppressed IL-2 production. The *in vitro* data show that conversion from DHEA to AED and from AED to AET is not accomplished in the mixed splenocyte culture, and mandates the conclusion that each of these steroid hormones has an independent effect on the control of cytokine secretion and cellular proliferation. Finally, the results show that even though AED and AET up regulate host resistance to infection there is a marked suppression of the inflammatory response and consequently, a protection from immune-mediated tissue injury.

SUMMARY

The physiological response to environmental challenge requires the rapid mobilization of the host functional capabilities as illustrated in the "fight or flight" reaction. This stress-mediated response is associated with a physiological increase in the level of ACTH and glucocorticosteroids. The acute phase response to infection and inflammation is also associated with an increase in glucocorticosteroid levels, which is required for the release of energy and metabolic functions. During subsequent stages of infection, the high levels of glucocorticosteroid may be necessary to clear the inflammatory infiltrate from target tissues and reduce inflammation. In juxtaposition, persistent and excessive stress with concomitant persistent and elevated glucocorticosteroid levels may result in immunosuppression and excessive pathology. Indeed, our experiments show that social isolation, physical (cold), or pharmacological (dexamethasone) stress all markedly augment pathology and mortality from virus encephalitis. The results presented herein, show that AET may function to upregulate the immune response. We propose that AET would counter the excessive downregulation of immune activation attributed to the excess production of cortisol. Figure 15.2 illustrates this concept and demonstrates the potential of AET as a neuroimmunomodulator. These findings suggest that the steroids AED and AET may in part function to balance the immunosuppressive effects of glucocorticoids.

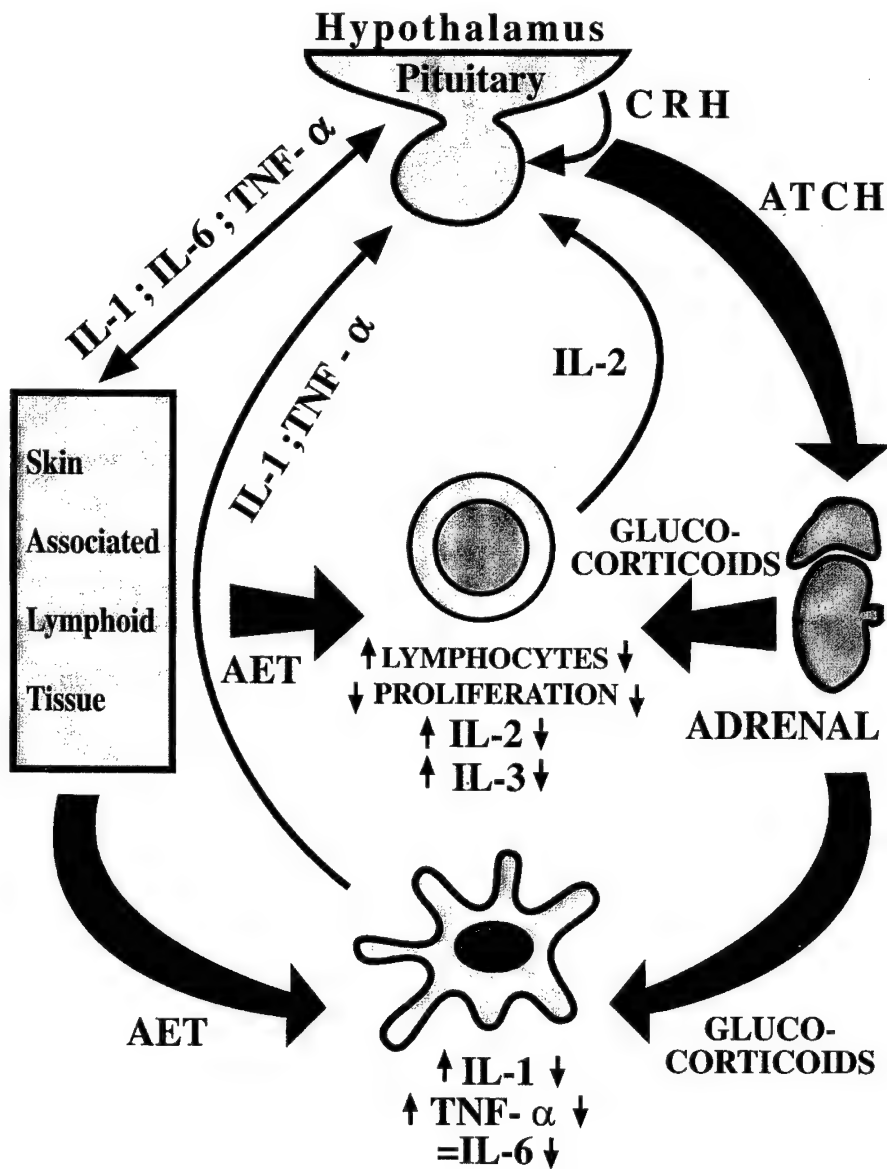


Figure 15.2 This diagram illustrates the possible roles of AET as a potential neuro-immunomodulator, which counteracts the effects of glucocorticoids on the immune system. IL-1, IL-2, IL-3 and IL-6 stand for the cytokines interleukin 1, 2, 3 and 6, respectively. TNF- α stands for tumor necrosis factor alpha. (Reproduced from Loria *et al.*, 1996. Regulation of the immune response by DHEA and its metabolites, by permission of the *Journal of Endocrinology*.)

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16 Neural, Endocrine, and Immune Mechanisms of Stress-Induced Immunomodulation

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Central nervous system (CNS) regulation of immunity is now a well-accepted concept (Savino *et al.*, 1995). The sympathetic nervous system innervates immune organs (e.g., thymus, spleen, bone marrow, and lymph organs), and immune organs and cells express receptors for catecholamine neurotransmitters released by the sympathetic terminals and adrenal medulla (Felten *et al.*, 1987). Furthermore, the sympathetic nerve terminals in these organs directly contact lymphocytes; these contact regions have ultrastructural features representative of synaptic contacts (Felten and Felten, 1991). In addition, lymphocytes and other immune cells express receptors for a variety of transmitters and hormones that are under the control of the CNS (Plaut, 1987). These include the hypothalamo-pituitary-adrenal (HPA) hormones CRH, ACTH, glucocorticoid, and β -endorphin.

Since stressors generally activate both the sympathetic nervous system and the HPA axis (indeed, stress is often defined in this fashion), it would be expected *a priori* that stressors should modulate immune function. There has been considerable interest in this interaction between stress and immunity, because it provides both a mechanism by which environmental events can alter the disease process and a window into relationships between the CNS, autonomic and endocrine systems, and the immune system. Numerous studies conducted over the past 20–30 years have demonstrated that a wide variety of stressors can alter many aspects of the immune response. In animals, acute exposure to electric shocks, separation, rotation, the odor of a stressed conspecific, immersion in cold water, restraint, handling, intraperitoneal injection of saline, loud noise, etc., have all been shown to suppress some aspect of immunity. Chronic stressors such as crowding have also been examined. In humans, acute stressors such as final examinations, battle-task vigilance, and sleep deprivation have been shown to impact on immune parameters. More chronic conditions such as divorce, bereavement, and Alzheimer caregiving also alter measures of immunity. Many of these studies have assessed some nonspecific aspect of immune function such as lymphocyte proliferation (Lylse *et al.*, 1988) or nitric oxide

(NO) release (Coussons-Read *et al.*, 1994) in response to mitogenic stimulation. More recently, studies have documented stress effects on specific immunity, such as the development of antibody to an antigen (Moynihan *et al.*, 1990; Fleshner *et al.*, 1989, 1992) or patterns of lymphocyte cytokine secretion in response to an antigen (Dobbs *et al.*, 1996). Stressors have also been shown to alter the migration pattern of immune cells between and into compartments of the immune system such as peripheral blood, spleen, thymus, lymph nodes, etc (Fleshner *et al.*, 1992, 1995b; Dhabhar *et al.*, 1996). Indeed, it is difficult to think of an aspect of immunity that has not been found to be altered by some stressor.

There are numerous excellent reviews of this literature (e.g., Kusnecov and Rabin, 1994), and only a few points can be noted here. First, the impact of stressors on immune function is highly variable, with reports of interference, facilitation, and no effect. The results depend on a host of variables such as the sex, age, experience, and genetics of the subject. In addition, the nature of the stressor and the immune measure studied are equally important. Stressors are not generic events, but they produce different neural and hormonal patterns depending on both physical attributes (e.g., duration, intensity, frequency), psychological dimensions (e.g., predictability, controllability) and subject characteristics (e.g., previous experience with the stressor, housing conditions, personality, social support). It should be remembered that the specific immune response extends over many days (antibody to an antigen first appears 3–4 days after exposure to the antigen), involving the continuous interaction of many different cell types. Thus, the effect obtained will naturally depend on the precise blend, duration, and timing of hormones and sympathetic activation by the stressor relative to the immune measure assessed. Second, the *mechanisms* that mediate the impact of stressors on immunity vary with the stressor and the immune measure. For example, Cunnick *et al.* (1990) found that the footshock-induced suppression of mitogen-induced proliferation of lymphocytes taken from the spleen is mediated by adrenal hormones, whereas the corresponding suppression in peripheral blood following the identical stressor is not.

INESCAPABLE SHOCK AND *IN VIVO* ANTIBODY

The foregoing suggests that an investigation of the mechanism(s) by which stressors alter immune function requires a choice of both the stressor used and the aspect of immunity measured. We have chosen a single session of inescapable tailshocks (IS) as the stressor and the *in vivo* production of antibody (Ig) to the antigen (Ag) keyhole limpet hemocyanin (KLH) as the measure of immune function. IS was chosen as the stressor because there is a wealth of knowledge concerning the neural and hormonal consequences of this stressor, and *in vivo* Ig to an Ag because it represents a natural end product of the specific immune response. In addition, *in vivo* measures allow the cells and organs of the immune system to remain in contact with the hormonal and other putative mediators of any effects of stress on immunity.

Our experiments (Fleshner *et al.*, 1992) indicate that there is roughly a 24-hour window around the administration of KLH during which exposure to IS (typically

80 1.0mA 5sec) suppresses the production of both IgM and IgG specific to KLH measured from blood samples taken from 5 to 30 days after the immunization. Furthermore, if rats are given a booster injection of KLH 57 days after the initial immunization, the subsequent secondary anti-KLH Ig response is also suppressed (Laudenslager *et al.*, 1988). Finally, this effect on *in vivo* Ig is not specific to IS and also follows defeat in aggressive encounters (Fleshner *et al.*, 1989).

T-HELPER CELLS

Figure 16.1 is a schematic of the sequence of events that intervene between immunization with Ag and the ultimate production of Ig. Exposure to IS could suppress the development of anti-KLH Ig by initiating a cascade that interferes at any of these steps. A series of studies has indicated that IS interferes with, at the earliest stages, the development of KLH-specific T-helper (Th) cells. These experiments have employed two-color flow cytometry to assess the development of the

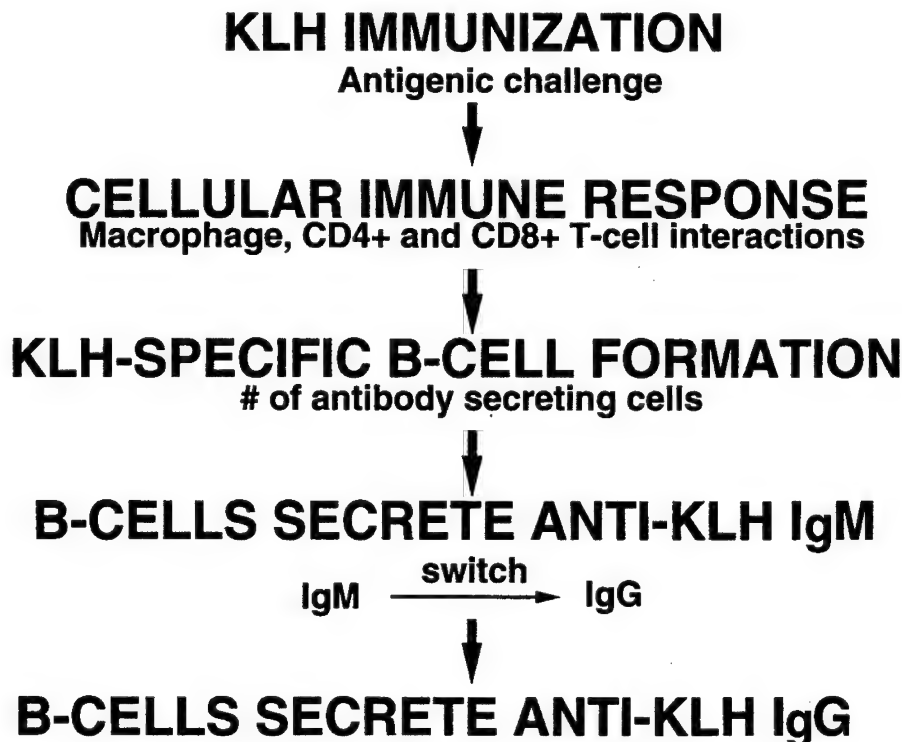


Figure 16.1 A simplified cascade of events following immunization with keyhole limpet hemocyanin (KLH). The first cellular events to occur is antigen presentation by antigen presenting cells (APCs). This in turn leads to an expansion of KLH-specific CD4+ T-cells (T4 or Th) and KLH-specific CD8+ T cells. KLH-specific Th cells help in the differentiation and proliferation of KLH-specific B cells. These cells first secrete IgM and then make an isotype switch and begin making IgG.

various different cell types after KLH and IS. Th cells express both the T-cell receptor (TCR) and the CD4 antigen, and so are designated TCR+CD4+. Figure 16.2A shows cell labeling after either IS or control treatment (homecage controls, HCC) 4 days after immunization with KLH or saline (SAL). Cells were taken from the draining lymph nodes (mesenteric nodes) and spleen. As expected, immunization with KLH increases the number of Th cells. These then are likely to be the KLH-specific Th cells that would be expected to develop 4 days after Ag. Exposure to IS clearly interfered with the development of these Th cells. In separate experiments IS given to nonimmunized rats had no effect on the number or percentage of Th cells in any compartment, supporting the idea that IS interferes with the expansion of KLH-specific Th cells. Furthermore, a reduction in TCR+CD4+ cells was not observed *immediately* after IS in immunized animals in any compartment (Fleshner *et al.*, 1992), again supporting the idea that IS prevents the KLH-induced *expansion* of Th cells in draining nodes and spleen, a process that requires 3–4 days.

There is evidence, therefore, that rats exposed to IS fail to expand CD4+ T cells in response to KLH (Fleshner *et al.*, 1995b). KLH is a T-cell-dependent antigen. Thus, diminished T cell help could be an important mechanism leading to a reduction in the formation of anti-KLH Ig. CD4+ T cells can be divided into two subsets, Th1 and Th2 cells, which differ in cytokine profile. The Th1 subset preferentially secretes interleukin-2 (IL-2) and interferon-gamma (IFN- γ), whereas the Th2 subset preferentially secretes IL-4 and IL-5. IL-2 is important for the proliferation of T cells, whereas IL-4 and IL-5 are important for the differentiation and proliferation of B cells. The Th1 and Th2 subsets of CD4+ T cells can be identified by the presence or absence of the cell-surface marker CD45RC (Powrie and Mason, 1990). We sought to identify which subset of Th cells were failing to expand to KLH in IS rats. Figure 16.2A shows the results of a study in which labeled cells were taken from the draining lymph node and spleen 4 days after immunization with KLH. Exposure to IS clearly prevented the expansion of KLH-specific Th1 cells. Th2 cell numbers were not effected at this time point after immunization. During the development of the anti-KLH immune response, anti-KLH Th1 cells develop first (within 3–4 days). The Th2 cells will often expand later using the IL-2 proliferative signal from the Th1 cells. Thus, we examined a later time point after immunization. Figure 16.2B shows that rats immunized with KLH and exposed to IS failed to expand the Th2 population of CD4+ T cells compared with KLH immunized HCC.

So, the pattern of the data indicate that IS interferes with Th1 development in the first few days after immunization so that cytokines necessary for Th2 development are reduced. Th2 development is therefore blunted, and the Th2 cytokines necessary for B-cell development and Ig secretion are reduced.

MACROPHAGES

IS could interfere with the expansion of KLH-specific Th cells in a variety of ways. A number of findings suggest that macrophages may play a key role. Macrophages often function as “natural suppressor cells” that inhibit a variety of lymphocyte functions, including cellular expansion (Mills, 1991). For example, infection of an

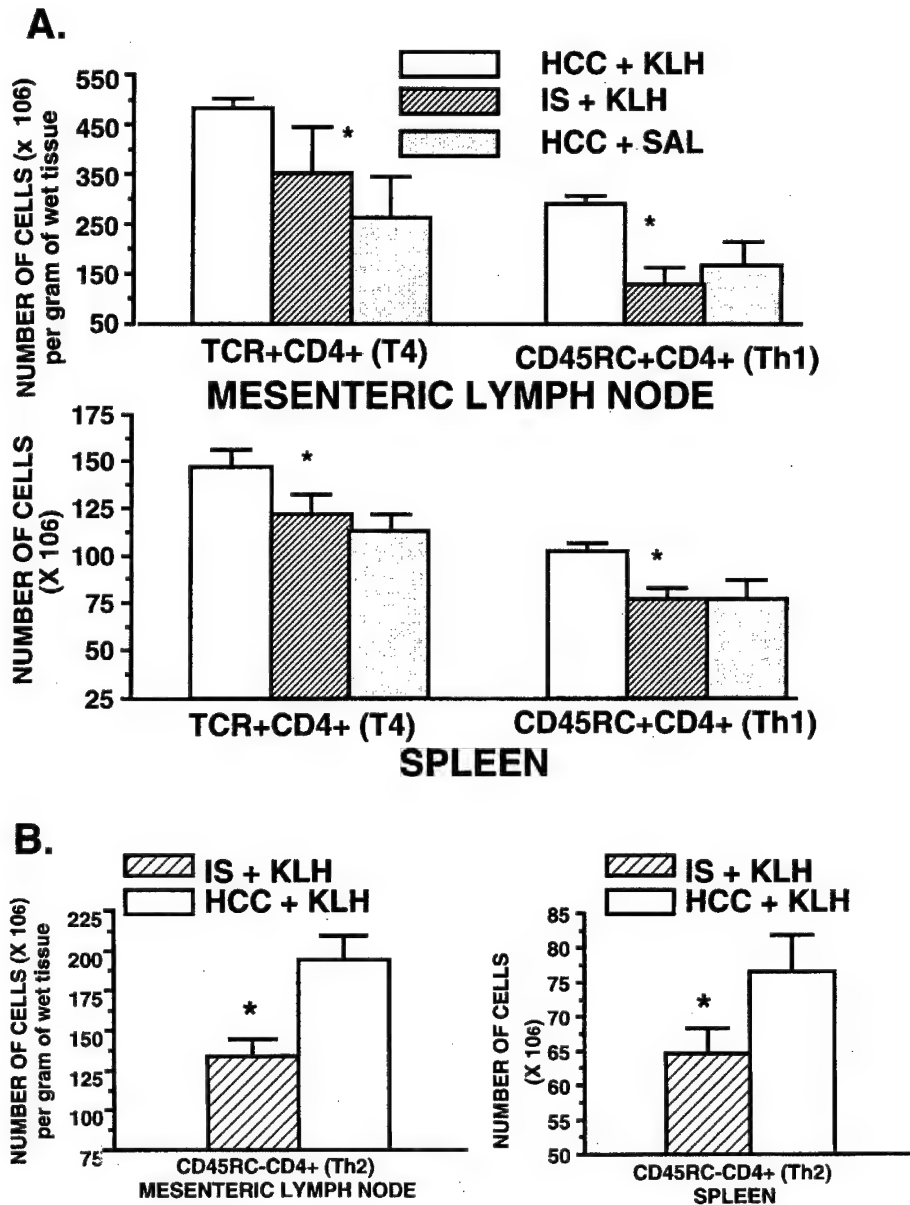


Figure 16.2 (A) Rats ($n=6/\text{grp}$) were either immunized with KLH (200 μg) and remained in their home cages (HCC+KLH), injected with saline and remained in their home cages (HCC+SAL), or were immunized with KLH and exposed to inescapable tail shock (IS+KLH; 100, 5-s, 1.6 mA). Four days after KLH+IS, CD4+ T cells and Th1 T cells were assessed using two-color flow cytometry. IS exposed rats failed to expand both CD4+ T-cells and Th1 cells in response to KLH. (B) Rats ($n=6/\text{grp}$) were either immunized with KLH (200 μg) and remained in their home cages (HCC+KLH), or were immunized with KLH and exposed to inescapable tail shock (IS+KLH; 100, 5-s, 1.6 mA). Ten days after KLH+IS, Th2 T-cells were assessed using two-color flow cytometry. IS exposed rats failed to expand the both CD4+ T-cells and Th2 cells in response to KLH.

organism with *Trypanosoma cruzi*, a protozoan parasite that activates macrophages, suppresses lymphocyte proliferation during the infection (Abrahamsohn and Coffman, 1995). This suppression has been shown to be produced by the activation of splenic macrophages and subsequent nitric oxide (NO) release. Activated macrophages secrete high levels of NO, a compound that interferes with T-cell proliferation via a number of mechanisms including a reduction in MHCII expression, IL-2 production, and apoptosis (Abrahamsohn and Coffman, 1995). Some stressors have been reported to produce NO release from cells in culture (Coussons-Read *et al.*, 1994) and blockade of NO synthesis can block the effects of a stressor on lymphocyte proliferation to mitogens (Coussons-Read *et al.*, 1994). Finally, inflammatory stimuli presented in close proximity to Ag interfere with the ultimate production of Ig to the Ag (Araneo *et al.*, 1989).

The foregoing led us to examine whether macrophages in subjects exposed to IS suppress the proliferation of Th cells. We did not wish to stimulate proliferation with mitogens because they bypass the normal signals generated by antigen binding to the T-cell receptor. Therefore, we instead examined the mixed lymphocyte reaction (MLR) because one can use culture conditions that specifically promote the proliferation of Th cells and the MLR requires antigen binding and signal generation by the T-cell receptor. In this procedure rats are exposed to IS or remain in their home cage. Immediately after IS, rats are sacrificed and the proliferation response of cells from the cervical lymph node and mesenteric lymph node in the MLR is measured. Exposure to IS resulted in a reduction in the proliferative Th response (Fleshner *et al.*, 1995a). To test whether the macrophages in culture were becoming suppressive to the proliferative Th response, they were removed from culture. Macrophage depletion blocked the IS-induced suppression of the MLR. To further test whether the IS-induced reduction in the MLR was due to macrophages, they were transferred from IS rats into the culture of home cage controls. The macrophages from the IS rats suppressed the MLR of the home cage controls, suggesting that the macrophage is indeed suppressive to Th proliferation (Fleshner *et al.*, 1995a).

NITRIC OXIDE

The experiments reviewed above suggest that IS activates macrophages, leading to the release of macrophage products that can interfere with the expansion of Th cells. Although a number of macrophage products can interfere with cellular proliferation (prostaglandins, transforming growth factor- β , NO), NO has received the greatest study. Thus we sought to determine whether IS does indeed lead to enhanced NO release from macrophages. Rats were exposed to IS or HCC and sacrificed 0, 48, or 96 hours after IS termination. Cells from the mesenteric lymph nodes and spleen were stimulated with ConA and Nitrite (the end product of NO) levels were measured in the supernatants. Figure 16.3 clearly shows that exposure to IS increases NO. The increase in NO is reliable immediately after IS termination in the mesenteric lymph node, the same time that we tested the MLR response for this lymphoid compartment. In contrast, NO levels were not reliably elevated in the spleen until 48 hours after IS termination, the time when Th cells are proliferating in response to KLH.

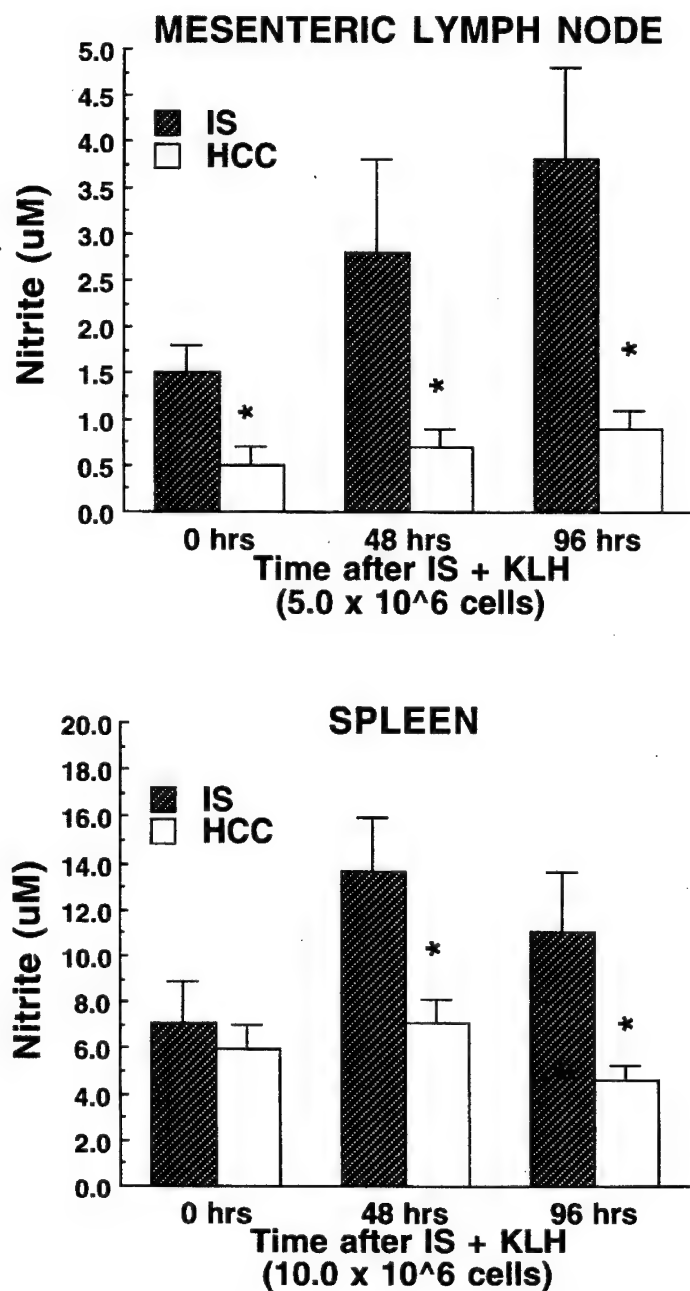


Figure 16.3 Rats ($n=6/\text{grp}$) were exposed to inescapable tail shock (IS) and were sacrificed immediately (0 hrs), 48 hrs and 96 hrs after IS termination. Home cage controls (HCC) were sacrificed at each time point. Nitrite was measured using the Griess Reaction from cultured (ConA, 48 hrs) mesenteric lymph nodes and spleen cells. IS exposure increased Nitrite accumulation in culture.

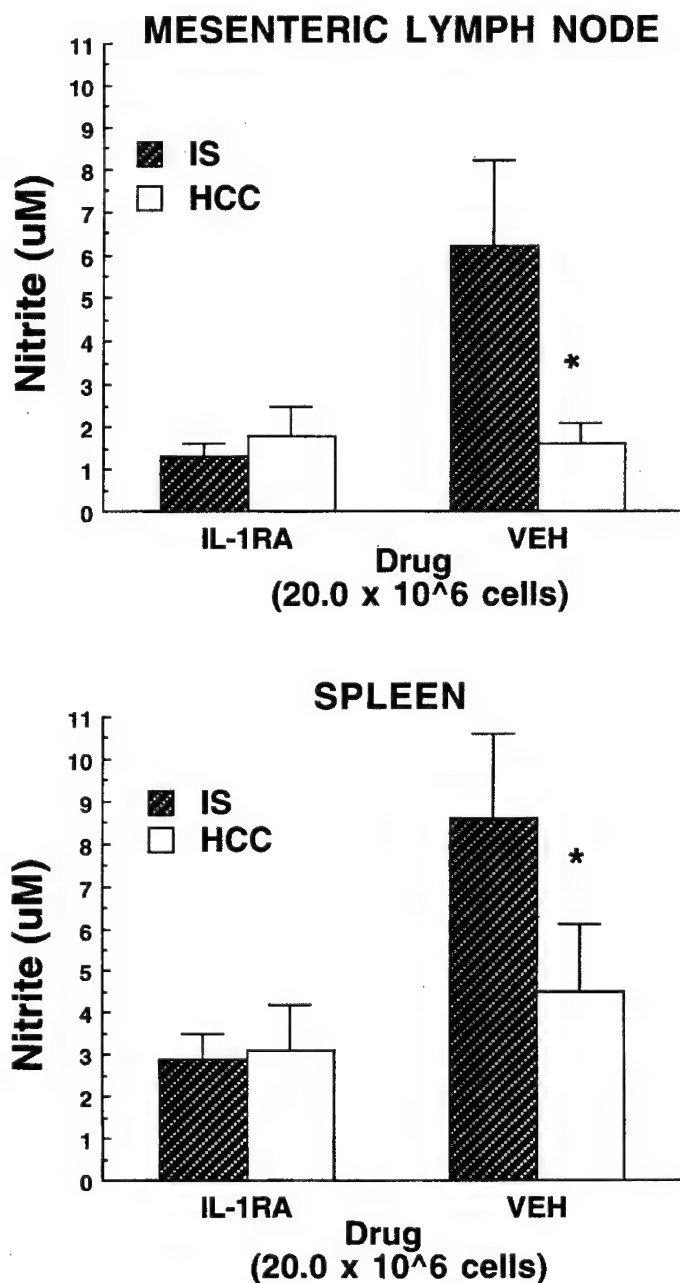


Figure 16.4 Rats ($n=6/\text{grp}$) were exposed to inescapable tail shock (IS) or remained in their home cages (HCC). Rats were then injected every 4 hrs for 24 hrs with IL-1 receptor antagonist (IL-1RA; 100 mg/kg) or vehicle (VEH). Four days after IS termination, rats were sacrificed and cells from the mesenteric lymph node and spleen were put in culture (ConA, 48 hrs). IS exposure increased Nitrite (Griess reaction) accumulation in culture and treatment with IL-1RA blocked this effect.

The fact that macrophages from IS animals release enhanced levels of NO upon stimulation does not prove that this NO is indeed responsible for the reduction of *in vivo* Ig. Clearly, experiments are required in which the enhanced NO production is blocked after IS and Ig to KLH measured. Activated macrophages also release IL-1, which is critical to NO production via an autocrine/paracrine action. We began by determining a dose of IL-1-receptor antagonist (IL-1ra) that would block the elevated NO production that follows IS. Figure 16.4 shows that administration of 100 mg/kg of IL-1ra every 4 hours for 24 hours after IS termination blocks the

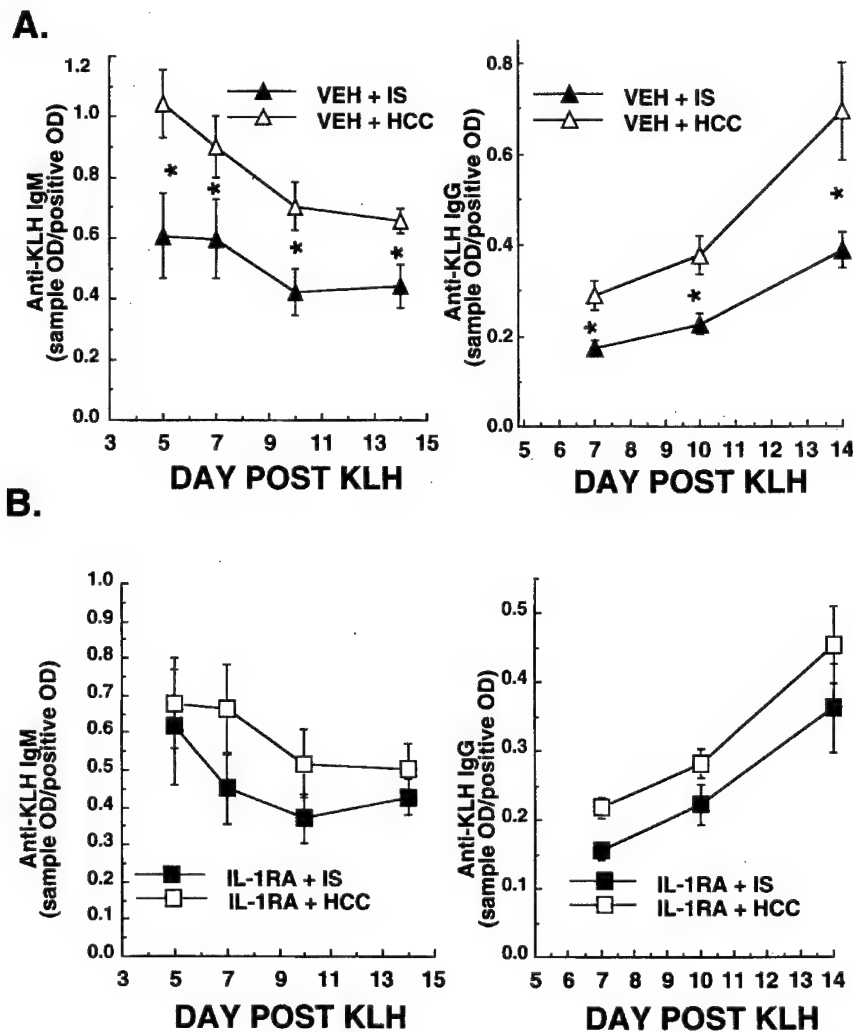


Figure 16.5 Rats ($n=12/\text{grp}$) were immunized with KLH and exposed to either inescapable tail shock (IS) or remained in their home cage (HCC). Rats were then injected every 4 hrs for 24 hrs with IL-1 receptor antagonist (IL-1RA; 100 mg/kg) or vehicle (VEH). Serum levels of anti-KLH IgM and IgG were measured. IS suppressed anti-KLH IgM and IgG. Treatment with IL-1RA block this effect.

IS-induced increase in NO. If the IS-induced elevation plays a role in the reduction in anti-KLH Ig produced by IS, then blocking the NO elevation should also block this Ig reduction. Figure 16.5B shows IL-1ra treatment (as described above) blocked the IS-induced suppression in anti-KLH Ig as compared to vehicle treated rats (Figure 16.5A). Thus if IS no longer resulted in elevated NO, then it also no longer suppressed anti-KLH Ig. In addition, activation of macrophages with a pharmacological agent (Zymosan) in the absence of IS, also resulted in both an increase in NO and a decrease in the anti-KLH Ig response (Fleshner *et al.*, 1996).

These data lend support to the idea that IS-induced suppression of the *in vivo* antibody response is caused by an elevation in NO, probably because NO elevation suppresses the expansion of KLH-specific Th1 cells which results in fewer Th2 cells. Fewer Th2 cells are then available to stimulate B cell proliferation and differentiation (Laudenslager and Fleshner, 1994) which results in a reduction in anti-KLH Ig.

CONCLUSIONS

These data suggest that IS interferes with the *in vivo* generation of Ig to an Ag administered within a 24-hour period around IS because IS induces macrophages to release NO and perhaps other products that inhibit the proliferation of cells in the region of the activated macrophages. This NO induction occurs 0–96 hours after IS, and this is the period during which the Ag-specific Th cells are developing.

Although this scenario may appear rather maladaptive, it should be appreciated that the specific immune response is the second line of defense against infection and invading pathogens. The antigenic specificity of the response requires an appreciable number of cell cycles in order to generate sufficient T and B cells bearing the receptor for the particular Ag to mount effector action (e.g., generation of cytotoxic T cells, Ab) against the Ag. Each cell cycle requires on the order of 8–12 hours, and so a number of days intervene before the specific immune response can act on an Ag after its first entry into the body. The innate immune response provides the first line of defense, and macrophage activation and macrophage products play a critical role in these processes. For example, NO inhibits pathogen replication as well as T-cell proliferation (Lorsbach and Russell, 1992), and the role of macrophage-derived cytokines in producing the acute phase response and sickness has received frequent attention (Baumann and Gauldie, 1994). Under ordinary circumstances the specific immune response may still proceed well enough that there is little cost to the reduction in Ig or other effector function that is produced by the innate immune mechanisms. It may be that it is only under the specialized circumstance in which a potent stressor (or broadly active inflammatory agent) occurs close in time to the first entry of an Ag that the inherent incompatibility between the two forms of defense lead to impaired immunocompetence.

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17 Stress and Cytokine–Brain Interactions

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The interrelationships between behavior, the brain, and the immune system are currently popular research areas (Maier *et al.*, 1994). Until very recently, the focus has been almost entirely on the flow of causation from psychological variables (e.g., exposure to a stressful event), to alterations in neural function, and finally to brain-induced changes in peripheral systems that can influence immune system organs and cells. Examples of such peripheral systems are the autonomic nervous system, which innervates and releases norepinephrine within immune organs such as the spleen and thymus (Felten and Felten, 1991), and the pituitary–adrenal axis, which releases hormones into the general circulation. The fact that immune cells express receptors for, and respond to, catecholamines, ACTH, corticosteroid, etc. (Plaut, 1987), provides a well-documented basis for how stress can dynamically modulate immune function.

However, it has become clear that the communication between the brain and the immune system is in fact bidirectional, and that products released from activated immune cells feed back and modulate neural activity, thereby modulating behavior, mood, and perhaps cognitive activity (Maier *et al.*, 1994; Watkins *et al.*, 1995). This is a very exciting concept, as immune-to-brain communication may be responsible for a large number of neural, behavioral, and affective alterations that are currently poorly understood. Thus this chapter will focus on two global concepts. First, the immune system, in addition to its classically defined functions in bodily defense, operates as a diffuse sense organ (Blalock, 1984; Blalock and Smith, 1985). As such, it monitors a number of key processes in the periphery and communicates their status to the central nervous system (CNS). This communication allows a number of early events in the body's response to infection or tissue damage to potentially modulate neural activity and behavior. Second, there is a striking degree of overlap in the biological machinery that is involved both in the bidirectional immune–brain pathways and in the classic stress response. This major overlap in pathways appears to be more than accidental.

IMMUNE-TO-BRAIN COMMUNICATION

Before one can understand how immune-to-brain communication occurs and why it is valuable, one must first grasp the cascade of events that result from infection or inflammation. When one is infected with a pathogen (such as bacteria, viruses, etc.), the immune system naturally responds by generating a specific immune response against the foreign invader. This occurs because cells of the immune system recognize the invader as "nonself". For example, T-lymphocytes with receptors for the particular "nonself" invader become activated and a chain of events ensues that ultimately results in the creation of antibodies specifically targeted against the encountered "nonself" antigen (Kuby, 1992). In reality, this process is slow, requiring a number of days before effective amounts of antibody are finally created. This time delay occurs because the specific immune response involves dramatic increases in immune cell proliferation, the sequential synthesis and release of substances that trigger and orchestrate each next step in the cascade, etc. (Kuby, 1992).

The emphasis is that this antigen-specific process takes a great deal of time and thus cannot serve as the body's first line of defense. Very rapid responses to infection/inflammation are also orchestrated by the immune system, but are not specific for the exact antigen encountered (Kuby, 1992). Rather, the rapid response is a generalized, nonspecific adaptive response called "sickness" (Hart, 1988; Kent *et al.*, 1992; Maier *et al.*, 1994). These immune-mediated events precede the antigen-specific response in time, appearing about 2 hours after infection/inflammation, and involve a constellation of behavioral and physiological changes that function to combat infection generally. This pattern is often called an Acute Phase Response (APR) (Kuby, 1992). The APR consists of: (a) fever; (b) shifts in liver synthesis away from normal products and toward the production of "acute phase proteins;" (c) alterations in plasma iron, zinc, and copper; (d) increases in circulating white blood cells; (e) changes in behavior, including increases in sleep, decreases in food and water intake, decreases in activity and exploration, decreases in social behavior and sexual behavior, decreases in aggression, and increases in pain reactivity (hyperalgesia); and (f) a classic stress response with increased release of pituitary-adrenal and sympathetic hormones (Hart, 1988; Kent *et al.*, 1992; Maier *et al.*, 1994; Watkins *et al.*, 1995).

What is notable about this list is that many of these changes after infection are mediated by the brain. Indeed, some (such as the behavioral changes) can only be produced by the brain. Not surprisingly, many of these responses to infection can be blocked by lesioning of, or administering antagonists into, appropriate brain structures (Rothwell and Luheshi, 1994). So, the peripheral immune cells that respond to the infectious agent must communicate with the brain. In support of this, both the electrical and chemical activity of the brain changes about two hours after infection, and these changes are quite specific to brain regions associated with illness-related outcomes (Saphier, 1989; Dunn, 1993). These are not simply reflexive responses to infection/inflammation, as animals are motivated to learn responses to allow illness-driven changes such as fever to occur (Kluger, 1991).

Perhaps it is easiest to place these widely diverse responses to illness in a framework with fever as the key response and the rest of the changes in service of

the generation of fever (Kluger, 1991). Fever is a phylogenetically very old and ubiquitous defensive response. Increased body temperature slows pathogen growth, accelerates some of the enzymatic processes involved in destroying pathogens, prevents the formation of bacterial protective coats, and is sufficient to itself destroy many bacteria and microorganisms. Fever accelerates the cellular proliferation of a number of immune cell types and is important for producing the alterations in plasma metals that affect pathogen growth. Therefore, fever is highly adaptive and numerous experiments show that reducing fever reduces survival (Kluger, 1991). But all of this is at a tremendous cost, given that creating and maintaining fever is very energy intensive: an approximate 10–15% increase in energy usage has been estimated for every degree of body temperature increase. So one may consider all other illness responses as either conserving energy necessary for generating fever (e.g., increased sleep, decreased social interaction, decreased energy expenditure associated with foraging) or as liberating energy from bodily stores (e.g., metabolic responses to glucocorticoids, norepinephrine). Only the CNS can orchestrate such diverse and widespread responses.

The next issue is how the immune cells actually signal the brain. The macrophage is critically important in the early response to infection/inflammation. These immune cells are attracted to the site of infection or injury and become activated. Activated macrophages release a myriad of products; of these, the proinflammatory cytokines (interleukin-1 [IL1], tumor necrosis factor [TNF], interleukin-6 [IL6]) are key mediators of immune-to-brain communication. These proinflammatory cytokines, as their names imply, orchestrate the immune system's early responses to infection/inflammation, including the APR. Blocking the actions of these proinflammatory cytokines also blocks the fever, pituitary–adrenal activation, behavioral, and other changes associated with infection/inflammation. Conversely, if you administer these substances, you create the entire APR without actual infection or inflammation (Maier *et al.*, 1994; Watkins *et al.*, 1995b,c). Of the proinflammatory cytokines, IL1 seems to be the most potent in these regards, and will be used as the prototypic example in the discussions that follow. One should note that parallel statements can be made of TNF and IL6 in most cases.

Thus, cytokines like IL1 must modulate neural activity. Originally, the thought was that IL1 accumulated at the site of injury/infection, spilled over into the blood, and was thus transported to brain (Watkins *et al.*, 1995b). In support of such an idea, IL1 brain receptors have been located and play a role in illness response. Injection of IL1 directly into brain initiates the APR, and blockade of brain IL1 receptors inhibits illness responses to systemically injected IL1 (Watkins *et al.*, 1995b). However, IL1 (and the other proinflammatory cytokines) are large proteins that are unlikely to cross the blood–brain barrier in concentrations substantial enough to exert effects. In response, investigators have proposed special mechanisms to transport the cytokines across the blood–brain barrier, including active transport, crossing at circumventricular structures that lack a tight blood–brain barrier, etc. However, the data substantiating these potential pathways are less than convincing (Watkins *et al.*, 1995b). Indeed, the most vexing fact has been that peripheral administration of amounts of IL1 too small to produce measurable blood levels still produce illness responses mediated by the brain (Kluger, 1991).

An alternative mechanism for immune-to-brain communication presents itself once one accepts the notion of the immune system as a diffuse sense organ. As such, one would then expect that information would arrive at the brain via peripheral nerves, just as it does for all other sensory systems. Using ip injections of IL1, TNF, and lipopolysaccharide (cell walls of gram-negative bacteria that activate immune cells to release IL1, TNF, and IL6), we and others have now shown that the vagus nerve conveys illness information to the brain (Watkins *et al.*, 1995b). Cutting the vagus blocks the ability of ip cytokines and lipopolysaccharides to produce fever, conditioned taste aversions, changes in brain monoamine levels, activation of the hypothalamic-pituitary-adrenal axis, decreases in feeding and drinking, decreases in social interaction, hyperalgesia, etc. (Watkins *et al.*, 1995b).

Anatomical studies have elucidated how IL1, at least, communicates to brain. IL1 binds to specialized structures called paraganglia, which are scattered along the course of the vagus nerve (Watkins *et al.*, 1995b). These paraganglia are structurally similar to chemoreceptive cells and synapse with (i.e., send information to) sensory fibers in the vagus. By this means, IL1 activates nerve fibers that carry the illness messages directly to the brain. The incoming illness messages are first communicated to the nucleus tractus solitarius, a key structure wherein the majority of vagal sensory fibers terminate (Ritter *et al.*, 1992). From here, messages are relayed to widespread brain structures that organize and carry-out the brain's response to illness cues, thus generating the APR (Ritter *et al.*, 1992).

But a puzzle remains. Earlier, the following points were raised which seem at odds with the notion of peripheral cytokines activating peripheral nerves to communicate to brain: (a) there are IL1 receptors in brain; (b) injecting IL1 directly into brain elicits the APR; and (c) IL1 antagonist injected into the brain blocks the APR that would normally follow systemic injection of IL1. These points strongly imply that cytokines do in fact act centrally to create the APR. The reconciliation of this apparent paradox is that the brain has cells (certainly glia and perhaps neurons) that manufacture the brain's own IL1 (Breder *et al.*, 1988; Saper and Breder, 1992; VanDam *et al.*, 1992; Yabuuchi *et al.*, 1994). That is, peripheral injections of IL1 drive *de novo* synthesis of IL1 in the CNS (Ban *et al.*, 1992; Gatti and Bartfai, 1993; Laye *et al.*, 1994). It is this IL1, synthesized and released by cells in the brain, that is the IL1 that is critical to the production of the brain-mediated changes leading to illness responses. To complete the bidirectional loop, Dantzer and his colleagues have recently shown that the vagus carries the signal from peripheral cytokines that leads to the induction of central IL1. Cutting the vagus blocked the induction of brain IL1 after peripheral lipopolysaccharide administration (Laye *et al.*, 1994).

IMMUNE-TO-BRAIN, AND THEN BACK AGAIN

One last point that warrants emphasis is that these communication pathways are bidirectional. IL1 in brain not only is involved in mediating CNS responses to illness (such as fever and behavioral alterations), but it also induces an outflow of hormonal and autonomic changes that can activate peripheral illness responses (such as shifts in liver protein synthesis). This is a bidirectional loop, with cytokines

being key players in both the periphery and brain. Thus, one should be, and indeed is, able to produce illness outcomes (e.g., hyperalgesia) by augmenting IL1 in either the periphery or brain (Watkins *et al.*, 1995c). IL1 administered either to the periphery or to the brain creates hyperalgesia; this is true for the other APRs, as well (Maier *et al.*, 1994). Continuing this logic, one should be able to block the effects of peripheral IL1 with IL1-antagonists delivered into brain; this is in fact true (Maier *et al.*, 1994). We have even shown that the effects of peripheral IL1 can be blocked by inhibiting the metabolic activity of glial cells in brain, the likely source of IL1 (Watkins and Maier, unpublished observations). Similarly, use of a drug that inhibits the synthesis of cytokines by glia also blocks illness responses (Watkins *et al.*, 1995a).

In summary, infection and tissue injury starts a cascade in the periphery that feeds to the CNS and back out again. Taken together, this bidirectional communication pathway regulates processes associated with defense against infection and inflammation.

OVERLAP OF STRESS AND IMMUNE-BRAIN PATHWAYS

So how does this relate to stress? Recall that part of the APR is the classic stress response with activation of both the hypothalamic-pituitary-adrenal axis and the sympathetic nervous system. Indeed, the constellation of effects generated during the APR (decreased eating, decreased social interaction, decreased locomotor activity, disturbances in sleep) may be described as characteristic symptoms of stress, depression, or anxiety. Indeed, the administration of IL1 produces depressed mood in humans (Maes *et al.*, 1993) and behaviors in laboratory animals characteristic of anxiety (Dantzer *et al.*, 1993).

One explanation in these commonalities between stress and illness is that they in fact activate some of the same circuits in the brain. This could occur, for example, if stressors lead to the synthesis and release of brain IL1. It would be natural to expect that there would be multiple ways to induce central IL1. If, as we are proposing, this is a true bidirectional system, it would not matter where one entered the loop, because the end results should be the same. One could predict then that stressed animals should behave like sick animals, which indeed they do. Using inescapable tailshock as an example from our own laboratory, it is clear that stressed animals *do* exhibit many of the same symptoms as sick ones: stress decreases food intake, water intake, social interaction, aggression, and locomotor activity as well as increasing pituitary-adrenal and autonomic activation (Peterson *et al.*, 1993). Recent work in our laboratory has also documented that further parallels exist between illness and stress, in that both create fever, identical shifts in liver protein synthesis, increases in circulating white blood cells, changes in blood ions, etc. Furthermore, it is now clear that stress activates some of the same brain pathways activated by illness. For example, both stress and infection deplete norepinephrine in the hypothalamus and both activate the nucleus tractus solitarius, where vagal sensory nerves first enter the brain.

If this schema is true, then it predicts that one could block the effects of stress by blocking IL1 in the brain. Indeed, blocking IL1 in the brain blocks both behavioral changes and fever resulting from inescapable tailshock (Maier and Watkins, 1995). As

for illness effects, stress-induced changes in behavior can be blocked by injecting a glial metabolic inhibitor into the brain (Watkins and Maier, unpublished observations). This again indicates that glia are the most likely source of IL1. Lastly, we now also know that stress does indeed increase IL1 (Nguyen, Fleshner, Watkins and Maier, unpublished observations) and its mRNA in discrete brain regions (Minami *et al.*, 1991).

Perhaps most surprising of all is that this does indeed appear to be a complete loop, as stressors actually activate macrophages just as illness does! We have examined three measures of macrophage activation (MHCII surface antigen expression, IL1 production of macrophages, and nitric oxide production by macrophages), and found that all dramatically increased, indicative of macrophage activation by a stressful event (Nguyen, Fleshner, Watkins and Maier, unpublished observations).

In summary, it appears inescapable stressors induce IL1 in brain, thereby producing many symptoms of illness, the stressor makes animals sick, and some of the sequelae of stress can be understood as manifestations of sickness occurring because stressors can tap into the circuitry that mediates illness. It is perhaps useful to reemphasize that administration of IL1 to humans leads to mood changes described as depression or anxiety, and that Maes has quite recently found that people with depression also show many symptoms of illness: a slight fever, high levels of acute phase proteins, etc. (Maes, 1995). Much more research needs to be done in this area, but the early evidence is indeed captivating.

But why is there such a striking overlap between: (1) the behavioral and physiological products of infection and the circuitry that mediates these effects; and (2) the products of stressors and the circuitry that mediates them? Any attempt at an answer must be by definition highly speculative. One potential explanation might be based on the fact that the syndrome that is called sickness functions at least in part to liberate energy from bodily stores and conserve its use for fighting infection. The stress response as classically defined is really a fight-flight response. It is important to recognize in this context that the processes involved in innate immunity (i.e., generalized macrophage responses to infection and tissue injury) are phylogenetically far older than the stress response or the fight-flight response. Even very primitive organisms need to defend themselves from pathogens and injury and possess these nonspecific mechanisms. The processes involved in specific immunity (T-cell recognition, antibody formation, and the like) are newer and built on top of these older mechanisms. The fight-flight response requires a more advanced organism since it requires detection of predators or threat at a distance, motor responses, and the ability to integrate the relevant sensory and motor processes to produce directed fight and flight. It also requires the liberation of large amounts of energy and the direction of the energy to a particular source — here, muscular exertion. Evolution seems to work by borrowing old parts to perform new functions, and there already was circuitry that would liberate energy and conserve it for a particular purpose. So, one could argue that the stress response rededicated the sickness machinery to a new purpose. All that was necessary was to now activate the machinery from a new source — external threat rather than a pathogen, and to make the necessary physiological adjustments so that the energy would go to muscles.

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18 Infection as a Stressor: The Role of Cytokines

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INTRODUCTION: PHYSIOLOGICAL CORRELATES OF STRESS

It is generally accepted that the most common physiological correlates of stress are a co-activation of the sympathetic nervous system and the adrenal medulla, and an activation of the hypothalamic–pituitary–adrenocortical axis (HPAA). The elevation of circulating catecholamines, discovered by Cannon (see Cannon, 1914), is considered to be fundamental to the “fight or flight” response, enabling mobilization of glucose and free fatty acids to provide energy, and directing blood towards the muscles and away from the viscera. The HPAA activation, discovered by Selye (see Selye, 1936) also mobilizes glucose, but the resulting increase in circulating glucocorticoids has a variety of other effects all geared towards enabling fight or flight. The other components of the HPAA, corticotropin-releasing factor (CRF), adrenocorticotropin (ACTH), and β -lipotropin (β -LPH) and β -endorphin each have their own independent effects (see Kramarck *et al.*, 1984).

These classical physiological responses in stress neglect the central nervous system (CNS) which must be critical in the decision to fight or flee as well as in adaptation to stress. Most experimental treatments regarded as stressful activate cerebral noradrenergic neurons as indicated by increased production of catabolites of noradrenaline (NA), increased extracellular concentrations of NA as determined by *in vivo* microdialysis or voltammetry studies, and after prolonged or intense stress, by depletion of NA (Dunn and Kramarck, 1984; Stone, 1975). This cerebral noradrenergic activation is considered to provide a central counterpart to the peripheral sympathoadrenal activation. Cerebral dopaminergic (DA) systems may also be activated during stress (Dunn, 1988a). The mesocortical DA system which projects to prefrontal and cingulate cortices is the most responsive, but DA projections to the neostriatum and nucleus accumbens are also affected (Dunn, 1988a). There is also substantial evidence that serotonergic systems in the brain are activated during stress, resulting in increased appearance of the serotonin

(5-hydroxytryptamine, 5-HT) metabolite, 5-hydroxyindoleacetic acid (5-HIAA: Dunn and Kramarcy, 1984), and in a few studies 5-HT determined by *in vivo* microdialysis or voltammetry (Kirby *et al.*, 1995). The changes in brain 5-HT metabolism appear to depend, at least in part, on increases in brain tryptophan (Curzon *et al.*, 1972), which are in turn dependent upon increased sympathetic nervous system activity, because ganglionic blockers and β -adrenergic antagonists prevent the CNS changes in tryptophan and 5-HIAA (Dunn and Welch, 1991). This increase in free brain tryptophan appears to be a consistent correlate of stress.

A substantial body of data indicates an important role for CRF in the brain during stress. CRF is found in neurons in a variety of extrahypothalamic brain regions in which it is unlikely to be related to the activation of the HPA. Intracerebral administration of CRF produces a broad variety of effects that resemble those observed in stress, including endocrine, electrophysiological, gastrointestinal, neurochemical, and behavioral effects (Dunn and Berridge, 1990). Endocrine consequences include HPA activation and inhibition of growth hormone and gonadotropin secretion, as well as sympathoadrenal activation. Electrophysiological consequences include desynchronization of the cortical electroencephalogram and activation of locus coeruleus noradrenergic neurons. Complex changes in gastrointestinal motility closely resemble those observed following other stressors. Intracerebral CRF potently activates noradrenergic and dopaminergic neurons, but not serotonergic ones (Dunn and Berridge, 1987). It also induces many behavioral effects, such as anorexia, changes in locomotor activity, increased grooming, decreased sexual and exploratory behavior, anxiogenic effects on conflict behavior, and a heightened responsivity to treatments that normally induce anxiety or fear responses. Thus, CRF administration mimics most responses commonly observed in stress (Dunn and Berridge, 1990; Owens and Nemeroff, 1991). That this role of CRF may be physiological is suggested because in many cases, intracerebral administration of peptide antagonists of CRF-receptors and antibodies to CRF attenuate or prevent the stress-related responses. Thus cerebral CRF has been suggested to be a central coordinator of responses in stress (Dunn and Berridge, 1990; Koob and Bloom, 1985; Owens and Nemeroff, 1991). These physiological correlates of stress are summarized in Table 18.1.

Table 18.1 Comparison of the physiological correlates of physical and behavioral stressors and infection with influenza virus

	<i>Physical and behavioral stressors</i>	<i>Influenza virus infection</i>
HPAA (plasma CRF, ACTH, β -endorphin and corticosteroids)	+++	+++
Sympathetic nervous system (plasma NA)	+++	+
Adrenal medulla (plasma A + NA)	+++	+
Brain NA (MHPG)	+++	+++
Brain DA (DOPAC)	+++	0
Brain 5-HT (5-HIAA)	++	++
Brain tryptophan	++	+++

ILLNESS AND STRESS

Because animal models of stress employ stressors that are not commonly experienced by humans, we examined the responses to infection with influenza virus, a stressor common to mouse and man. The results showed that mice infected with influenza virus by direct infusion of the virus into the lungs showed the behavioral and body weight changes typically associated with infection by about the second to third day. Progressive increases in plasma concentrations of ACTH and corticosterone were observed until the mice succumbed to the disease on around the sixth to seventh day (Dunn *et al.*, 1989). When neurochemical analyses were performed, 3-methoxy, 4-hydroxyphenylethyleneglycol (MHPG), a major catabolite of NA, was markedly elevated, with increases in the hypothalamus observed within two days, and in other brain regions by the fourth day. The metabolites of DA were not significantly altered. Brain tryptophan and 5-HIAA were elevated within 2–3 days, but these indolaminergic changes were not regionally selective (Dunn *et al.*, 1989). The remarkable parallels between the pattern of changes associated with influenza virus infection and commonly used laboratory stressors, such as footshock or restraint, is shown in Table 18.1. The parallel activation of peripheral and central noradrenergic systems and the HPA axis is striking. The major differences were that in the influenza virus-infected animals, the noradrenergic activation was consistently greater in the hypothalamus, and that changes in brain DA metabolism were not observed. Table 18.2 indicates the behavioral parallels between the consequences of influenza virus infection and standard laboratory stressors. The remarkable similarity in the physiological, neurochemical and behavioral consequences suggests that similar or common mechanisms may be involved.

A ROLE FOR CYTOKINES

An important clue to a potential mechanism for the responses to infection, was the discovery that administration of the purified cytokine, interleukin-1 (IL-1), at very low doses to rats elicited a marked HPAA response. Plasma ACTH and corticosterone concentrations were elevated in a dose-dependent manner (Besedovsky *et al.*, 1986). Interleukin-1 is a protein produced by a variety of immune cells early

Table 18.2 Behavioral effects of stressors, infection with influenza virus or interleukin-1 treatment

<i>Behavioral response</i>	<i>Physical and behavioral stressors</i>	<i>Influenza virus infection</i>	<i>Interleukin-1</i>
Hyperthermia (fever)	+	+	+
Locomotor activity	—	—	—
Feeding	—	—	—
Exploratory activity	—	—	—
Sexual behavior	—	—	—
Withdrawal	+	?	+
Slow-wave sleep	?	+	+

during immune activation and plays an important role in marshalling an effective T-cell response. We replicated the IL-1-induced HPAA activation in mice, but most interestingly, the IL-1 administration also elicited increases in brain MHPG, tryptophan and 5-HIAA (Dunn, 1988b). The results paralleled those obtained with influenza virus remarkably. The MHPG increase peaked at 2 hours, and although present in all brain regions examined, was significantly greater in the hypothalamus. No changes were observed in DA metabolism. And, the increases in tryptophan and 5-HIAA were not regionally selective and were significantly delayed, peaking around 4–8 hours. A very similar pattern of responses was observed following administration of endotoxin (lipopolysaccharide, LPS: Dunn, 1992a) or Newcastle disease virus (NDV: Dunn and Vickers, 1994), two agents known to be potent stimulators of the immune system. Thus the results suggested that IL-1 was responsible not only for the HPAA activation, but also for the neurochemical responses. These results supported the proposal of (Besedovsky *et al.*, 1986) that IL-1 signals the brain regarding the activation of the immune system stimulating the HPAA, and that other cytokines may act similarly (Figure 18.1).

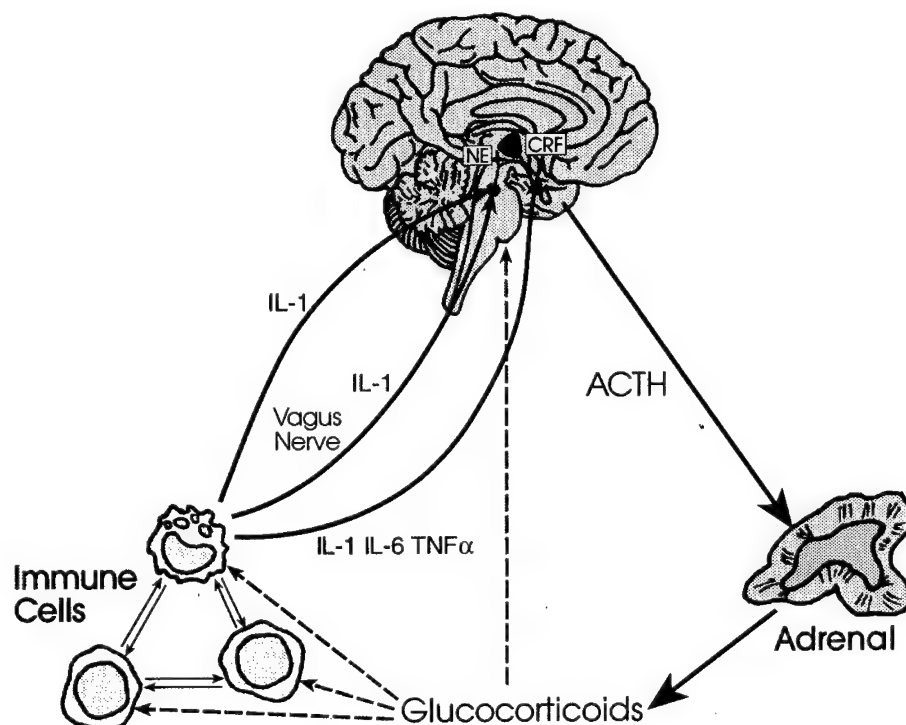


Figure 18.1 The Cytokine Loop. Immune activation results in the secretion of cytokines, such as IL-1, IL-6 and TNF α . These cytokines then act directly or indirectly (e.g., via the vagus nerve) on the brain to activate noradrenergic neurons in the brain stem that project to the hypothalamic paraventricular nucleus (PVN). The PVN CRF-containing neurons secrete CRF in the median eminence region of the hypothalamus initiating the activation of the HPAA, causing secretion of ACTH (and β -LPH and β -endorphin) from the anterior pituitary, in turn causing corticosteroid secretion from the adrenal cortex. The adrenal corticosteroids are known to inhibit immune activation providing a negative feedback mechanism on the immune system.

INTERLEUKIN-1-INDUCED HPAA ACTIVATION

The mechanism of the HPAA activation by peripherally administered IL-1 was shown to resemble that involved for other stressors. The pituitary was required because hypophysectomized mice failed to show any plasma corticosterone response to IL-1 (or LPS or NDV; Dunn, 1993b; Dunn and Vickers, 1994). Similarly, a requirement for CRF was indicated because pretreatment with an antibody to CRF largely prevented the plasma ACTH and corticosterone responses to IL-1 (Dunn, 1993b). Because a substantial body of data suggests a role for hypothalamic NA in regulating CRF secretion from paraventricular neurons, an interesting question was whether the noradrenergic activation associated with IL-1 administration was instrumental in eliciting the HPAA response. Experiments with the α_1 -adrenoreceptor antagonist, prazosin (1 mg/kg) showed about a 50% reduction in the increases in plasma ACTH and corticosterone normally induced by IL-1 in mice. However, in rats, noradrenergic lesions of the ventral ascending noradrenergic bundle or the paraventricular nucleus (PVN) of the hypothalamus indicate a substantial loss of responsivity of plasma corticosterone to intraperitoneal (ip) IL-1 (Chuluyan *et al.*, 1992). Microdialysis studies showed an increase of extracellular NA in the hypothalamus concomitant with the elevation of plasma corticosterone following intravenous (iv) or ip IL-1 (Smagin *et al.*, 1996). These data all suggest that hypothalamic NA is involved in the IL-1-induced activation of the HPAA, although other factors may also contribute.

THE CATECHOLAMINERGIC AND SEROTONERGIC RESPONSES ARE DISTINCT

All the above studies indicated a close parallel between the cerebral noradrenergic activation and that of the HPAA. The indolaminergic changes correlated less well, such that the peak responses to IL-1 and LPS in brain tryptophan and 5-HIAA were considerably later than those of MHPG and the HPAA (Dunn, 1992a; Lavicky and Dunn, 1995). When the responses to LPS were studied in endotoxin-resistant mice, the HPA response was markedly attenuated and the MHPG response was absent, but the indolaminergic responses were unimpaired (Dunn and Chuluyan, 1994). Responses to IL-1 were normal. Contrariwise, when mice were pretreated with a nitric oxide synthase inhibitor (N ω -nitro-L-arginine methyl ester), the indolaminergic responses were prevented, while no impairment was observed in the HPA and noradrenergic responses (Dunn, 1993a). These results indicate that different mechanisms must be involved in the catecholaminergic and indolaminergic responses to LPS and IL-1, and similar data suggest this is also true for other stressors.

THE ROLE OF INTERLEUKIN-6 (IL-6)

IL-6 is a second cytokine that is produced relatively early in the immune response, and increased plasma concentrations of this cytokine have been reported in a variety of disease states (including influenza virus infection (Hennet *et al.*, 1992) and LPS treatment (Coceani *et al.*, 1993). Administration of mouse IL-6 to mice elicits a

Table 18.3 Physiological correlates of influenza virus infection and cytokine administration

<i>Stimulus</i>	<i>ACTH/Corticosterone</i>	<i>NA</i>	<i>Tryptophan</i>	<i>5-HT</i>	<i>DA</i>
*Flu infection	+	+	+	+	0
LPS	+	+	+	+	+
NDV	+	+	+	+	0
IL-1	+	+	+	+	0
IL-6	+	0	+	+	0
TNF α	+	?	0	0	0

short-lived activation of the HPAA, perhaps due to the short plasma half-life of this cytokine (Dunn and Wang, 1996). This treatment elicited very little change in catecholamine metabolism, but brain tryptophan and 5-HIAA were increased at relatively low doses (0.25 μ g per mouse) (Dunn and Wang, 1996). Thus, this cytokine may contribute to the HPAA activation by some immune stimuli, and may be responsible in part for the indolaminergic response to IL-1 and LPS, both of which increase circulating concentrations of IL-6. A summary of the responses to various immune stimuli and cytokines appears in Table 18.3.

THE ROLE OF IL-1 IN THE BEHAVIORAL RESPONSES TO INFLUENZA VIRUS INFECTION

The working hypothesis is that cytokines produced by cells of the immune system mediate the nervous system responses to infections (Figure 18.1). Studies with the IL-1-receptor antagonist (IL-1ra) have only partially supported this hypothesis. IL-1ra treatment failed to alter the HPA and neurochemical responses to LPS (Dunn, 1992b; Dunn and Brown, 1996), although it partially prevented the responses to NDV (Dunn and Vickers, 1994). Likewise, an antibody to tumour necrosis factor α (TNF α), another cytokine induced by immune system stimulation, failed to block the HPA and neurochemical responses to LPS, even in combination with IL-1ra (Dunn, 1992b).

As indicated above, influenza virus infection induces hypomotility and anorexia (Swiergiel *et al.*, 1997). These effects were not reversed by treatment with indomethacin. They were, however, mimicked acutely by administration of IL-1 or LPS (Swiergiel *et al.*, 1997). Therefore, we tested the ability of IL-1ra to alter these behavioral effects of influenza virus infection. IL-1ra treatment was able to prevent the responses to IL-1, and to attenuate those to LPS. However, IL-1ra treatment did not prevent the anorexic or hypomotility effects of influenza virus infection (Swiergiel *et al.*, 1997). Interestingly however, chronic IL-1ra treatment (delivered by osmotic pumps) did reduce the mortality of mice infected with influenza virus.

CONCLUSIONS

The above brief review indicates that infections can induce physiological and behavioral responses like those to physical and behavioral stressors. They activate

the HPA and the sympathoadrenal system, elevating circulating concentrations of ACTH, corticosterone, and catecholamines. They activate cerebral catecholaminergic and serotonergic systems, although there are some differences in that infections do not typically activate dopaminergic systems, the noradrenergic responses are focussed on the hypothalamus, and the indolaminergic responses are delayed. Behavioral similarities include the induction of hyperthermia, hypomotility, anorexia, decreased libido, and a decreased interest in exploring the environment, although infections also increase slow-wave sleep.

The mechanism of the immune activation of the brain appears to involve the actions of cytokines produced by cells of the immune system and active on the brain, but although cytokines, such as IL-1, can mimic most of these effects, they do not appear to be the only factor(s) involved in the responses. Very low doses of IL-1 administered peripherally (or centrally) activate the HPA and the sympathoadrenal system (the latter less effectively). The mechanism of HPA activation largely involves hypothalamic CRF and requires an intact pituitary and adrenal gland. Low doses of IL-1 also activate noradrenergic systems especially in the hypothalamus, which may mediate the HPA activation. They also induce a delayed elevation of brain tryptophan, and activate serotonin metabolism. IL-1 also induces a number of behavioral effects, hyperthermia, hypomotility, anorexia, decreased libido and interest in exploring the environment, as well as increased slow-wave sleep. Thus IL-1 alone can account for many of the neurochemical, endocrine and behavioral effects of infections. Certain other cytokines, such as IL-6 and TNF α have some similar effects. However, experiments with cytokine antagonists have been notably unsuccessful in reversing many of the effects of infections, suggesting that multiple mechanisms (cytokine or other) are involved.

The results suggest that infections activate behavioral and physiological reactions very similar to other stressors studied, and that similar CNS mechanisms are involved.

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19 Stress, Natural Killer Cell Activity, and Tumor Metastasis: The Role of Catecholamines and Corticosteroids

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Stress is known to affect immunity and tumor development. We describe several approaches for assessment of natural killer (NK) cell activity, and report the effects of three stress paradigms on NK activity and on the susceptibility to tumor development. Our findings indicate causal relationships between stress and suppressed NK cytotoxicity, as well as between this suppression and increased tumor metastasis. Activation of the sympathetic nervous system and the release of adrenal catecholamines are suggested to underlie these stress effects. Whereas high physiological levels of corticosterone induced by stress or by systemic administration appear not to be a sufficient or a necessary condition for the suppression of NK activity, *in vivo* administration of adrenaline or metaproterenol, a β -adrenergic agonist, caused a marked suppression of NK activity and enhanced tumor metastasis. These adrenergic effects appear to be mediated via activation of peripheral mechanisms.

INTRODUCTION

The immune system is considered to be an important channel through which psychological factors affect health. Several hormones and peripherally released neurotransmitters have been shown to affect immune cells, and innervation of immune organs is thought to play a role in altering immune function. Most hormones associated with stress responses have been implicated in immune modulation. Cellular mechanisms for this immune modulation include altered leukocyte expression of surface proteins important for recognition, adherence or cytotoxicity, and altered production of humoral factors such as cytokines, cytotoxic factors, and antibodies (Ader *et al.*, 1991).

Natural killer (NK) cells, an important component of innate immunity, are a subpopulation of lymphocytes capable of recognizing and killing malignant and virally-infected cells without prior sensitization (Herberman *et al.*, 1981; Brittenden *et al.*, 1996). NK cells are especially important in controlling metastatic growth of various malignancies (Barlozzari *et al.*, 1985; Ben-Eliyahu *et al.*, 1992; Wiltrout *et al.*, 1985; Brittenden *et al.*, 1996).

Stress and catecholamines have been reported to cause alteration in NK activity (Kappel *et al.*, 1991; Schedlowski *et al.*, 1993; Tønnesen *et al.*, 1987) and in adherence and redistribution of various leukocyte subpopulations, including NK cells (Benschop *et al.*, 1993). NK activity is usually assessed per a constant number of leukocytes, monocytes, or splenocytes. Stress affects the numbers of these cells in various immune compartments (e.g., blood, spleen) independently of its effects on the effect of NK cells. It is therefore important to determine whether stress affects NK cytotoxicity by altering the activity per NK cell, or by altering the number of NK cells within the cell population tested for cytotoxicity. Most of our studies reported here assessed both the number and activity of blood NK cells.

The effects of stress on NK cell number and activity, the impact of these changes on susceptibility to tumor development, and the agents mediating these effects were studied in our laboratory and are the subject of this review.

THREE COMPLEMENTARY APPROACHES FOR THE STUDY OF NK ACTIVITY

The NK cytotoxicity assay measures the ability of NK cells to kill tumor cells *in vitro*. To study the effects of stress on NK activity, and the role of NK cells in mediating the effects of stress on malignant development, we have used several approaches. First, we have conducted *ex vivo studies*: NK activity was assessed *in vitro* following *in vivo* manipulations such as drug administration or exposure to stress. Using this *ex vivo* approach, NK cells are exposed to the overall complex changes induced by stressors in the *in vivo* milieu. NK activity, however, is assessed thereafter in a standard *in vitro* environment in the absence of many physiological factors that are washed away (e.g., hormones), or are absent (e.g., epithelial cells). Second, we have conducted *in vitro studies* in which stress hormones are added to the standard medium of the *in vitro* assay. NK activity is assessed in the presence of hormones and good control over the variables affecting NK activity is achieved. Nevertheless, the effects of hormones are studied in the limited and artificial context of the *in vitro* environment. Neither approach indicates the biological significance of changes in NK activity.

In consideration of these factors, we have also used a third approach: *in vivo assessment of NK activity* in which an NK-dependent process is evaluated in the living rat. The process assessed is clearance of MADB106 tumor cells from the lungs following intravenous inoculation, and prevention of the development of lung metastases. The MADB106 tumor line is syngeneic to the inbred F344 rats used in this study and metastasizes only to the lungs, thus constituting a convenient model of breast cancer metastasis. The extent of the metastatic process is indicated by the retention of tumor cells in the lungs 24 hr after tumor inoculation, or by the number of lung metastases counted three weeks later. These indices are largely dependent on the activity levels of NK cells during the first 24 hr after tumor injection, but not later (Barlozzari *et al.*, 1985; Ben-Eliyahu *et al.*, 1991, 1992). Therefore, the host antimetastatic activity reflects *in vivo* levels of NK cell activity in a manner pertaining to its biological significance.

THE EFFECTS OF STRESS ON NK ACTIVITY AND TUMOR DEVELOPMENT

Stress Paradigms

We have used three stress paradigms: (a) swim stress, in which the rat goes through 5 cycles in which it swims for 3 min and rests for 3 min (for a total of 30 min). Water is maintained at 37°C and a weight of 45 g/kg is attached to the rat's tail (Ben-Eliyahu *et al.*, 1991, 1992); (b) laparotomy stress, in which a 4 cm midline incision is made in the abdominal muscle wall under halothane anesthesia. The small intestine is externalized, rubbed, and returned to the abdominal cavity, then the skin and muscle layer are sutured (Page *et al.*, 1993, 1994); and (c) social confrontation stress, in which a male intruder is introduced into a cage accommodating an established male-female colony (Stefanski *et al.*, 1996).

Ex Vivo Studies

Stress was shown to suppress NK activity in several *ex vivo* studies. One hour following swim stress, splenic NK cytotoxicity against both the YAC-1 and the syngeneic MADB106 tumor line was suppressed, and this suppression was found to be naloxone insensitive (Ben-Eliyahu *et al.*, 1990, 1991). NK activity per ml blood was suppressed at 20 min, and 1, 3, but not 7 hr following swim stress. Laparotomy stress suppressed splenic and blood NK cytotoxicity against both YAC-1 and MADB106 tumor cells throughout the first day following surgery (Page *et al.*, 1992, 1994). In both stress paradigms, the number of blood NK cells (NKR-PI⁺ bright) was either unaffected or increased (at the time points NK activity was assessed) (Ben-Eliyahu *et al.*, 1993; Page *et al.*, 1994), thus indicating reduced NK cytotoxicity per blood NK cell.

In Vivo Studies

Swim stress, laparotomy, and social confrontation caused a 2 to 5 fold increase in MADB106 lung tumor retention (Ben-Eliyahu *et al.*, 1993; Page *et al.*, 1993; Stefanski *et al.*, 1996) and a similar increase in the number of lung metastases counted three weeks later (Ben-Eliyahu *et al.*, 1991; Page *et al.*, 1993). Swim stress also increased the mortality rate from the CRNK-16 leukemia line. This line is known to be NK/LAK sensitive and is syngeneic to the F344 rats. For the above studies, tumor cells were injected in close time association with the stress period: 1 hr after swim stress, 4–6 hr after laparotomy, and 1 hr after the beginning of an 8-hr social confrontation session.

A Role for NK Cells in Mediating Stress Effects on Tumor Development

Various *in vivo* factors that are affected by stress may underlie alterations in susceptibility to tumor development. Determining the relative role of NK cells in mediating such effects, although difficult, is important for evaluating the biological significance of stress-induced alterations in NK activity assessed *ex vivo*. We have used

several approaches to address this question. First, employing swim stress and laparotomy, we have demonstrated an identical time course between stressor-induced NK suppression (*ex vivo*) and stressor-induced increases in metastatic development (*in vivo*): Swim stress induced both changes at 1, but not 7 hr following stress, and laparotomy during the first day after surgery but not later (Page *et al.*, 1992, 1993). Second, NK activity controls the number of MADBI06 lung metastases during the first 24 hr following tumor inoculation, but not later (Ben-Eliyahu *et al.*, 1992; Barlozzari *et al.*, 1985). Similarly, swim (Figure 19.1) and laparotomy stress increases number of lung metastases when employed within less than 24 hr of tumor inoculation, but not later (Ben-Eliyahu *et al.*, 1991; Page *et al.*, 1993).

Third, we have used selective *in vivo* depletion of NK cells (using the mAb 3.2.3) and compared the effects of stress on tumor metastasis between normal and

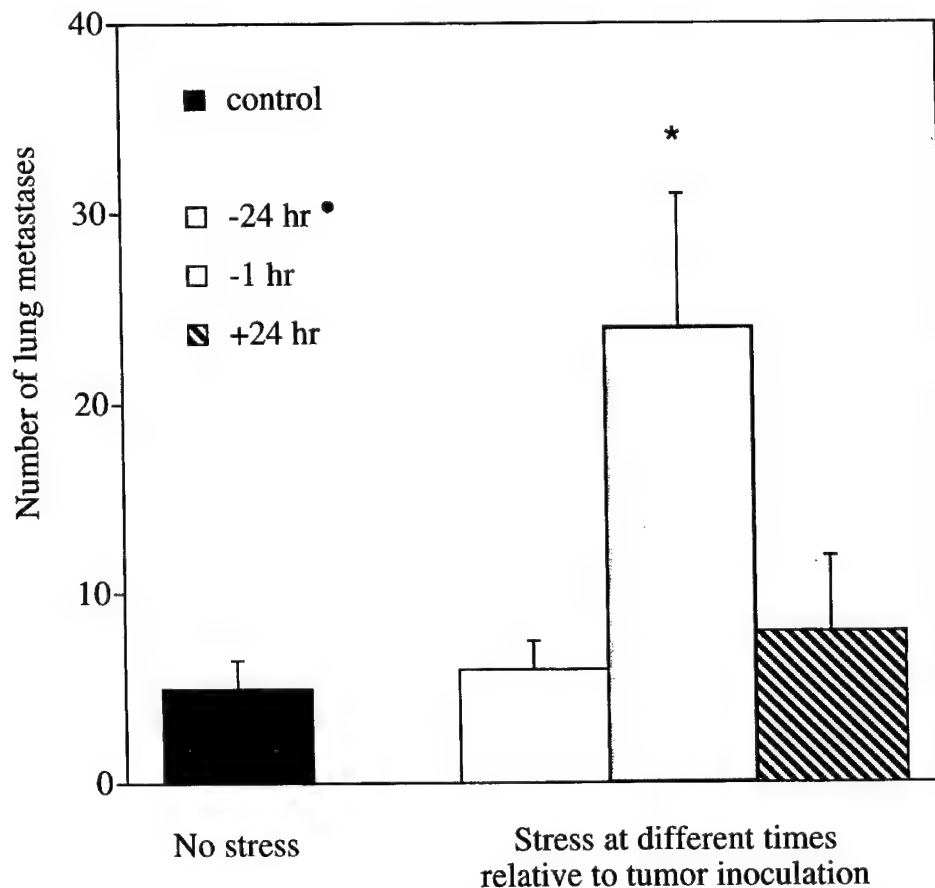


Figure 19.1 The effects of swim stress on the number of lung metastases. Error bars represent SEM. * — significantly different from control.

NK-depleted rats. If the effects of stress are mediated exclusively by NK cells, stress should not affect tumor metastasis in NK-depleted rats. Whereas laparotomy stress increased tumor retention in both normal and NK-depleted rats, swim stress, having a larger effect than laparotomy in normal rats, had no effect in NK-depleted rats (Ben-Eliyahu *et al.*, 1993) (Figure 19.2). These findings indicate a major role for NK cells in mediating the effects of swim stress on metastasis of the MADB106 tumor, but not an exclusive role for NK cells in the case of laparotomy stress.

Last, we compared the effects of swim stress on lung tumor retention of two different lines of tumor cells that metastasize to the lungs: the NK-sensitive MADB106 line, and the NK-insensitive (Ben-Eliyahu *et al.*, 1996), C4047 colon tumor line. Increased tumor retention was evident only in rats injected with the NK-sensitive line.

Taken together, this evidence suggests that NK cells play a critical role in mediating the effects of swim stress on the metastasis of MADB106 cells.

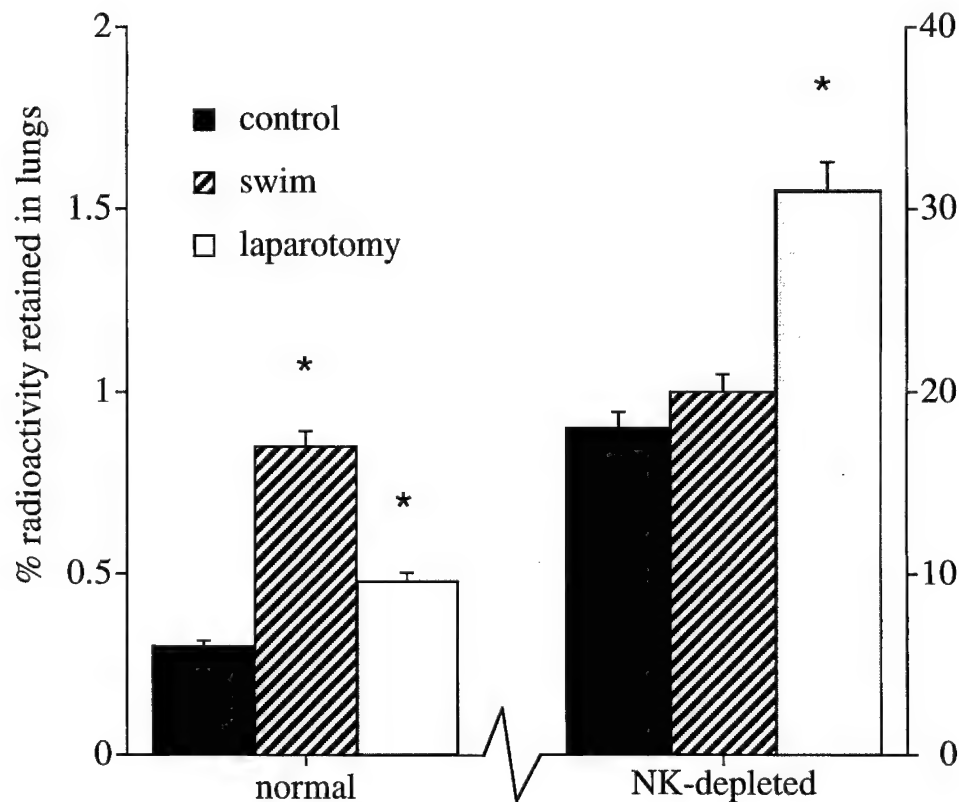


Figure 19.2 The effects of swim stress and laparotomy on lung tumor retention in normal and NK-depleted rats. Error bars represent SEM. * — significantly different from its respective control.

A ROLE FOR β -ADRENERGIC MECHANISMS IN THE SUPPRESSION OF NK ACTIVITY

Preventing the Effects of Stress by Sympathetic Blockade

We were able to block the tumor-promoting effects of swim stress by employing three methods: (a) administration of the ganglionic blocker chlorisondamine (3 mg/kg), (b) adrenal-demedullation (Figure 19.3), and (c) administration of the β -blocker butoxamine (25 mg/kg). A 2×2 design was used in each experiment: stress/no-stress by treatment/no-treatment. Each treatment abolished or reduced the stress-induced increase in the number of MADB106 lung metastases without exerting significant effects in nonstressed rats. Butoxamine and adrenal-demedullation had similar effects when lung tumor retention was assessed (Ben-Eliyahu *et al.*, 1993). Butoxamine also reduced the tumor-enhancing effects of social confrontation (Stefanski *et al.*, 1996).

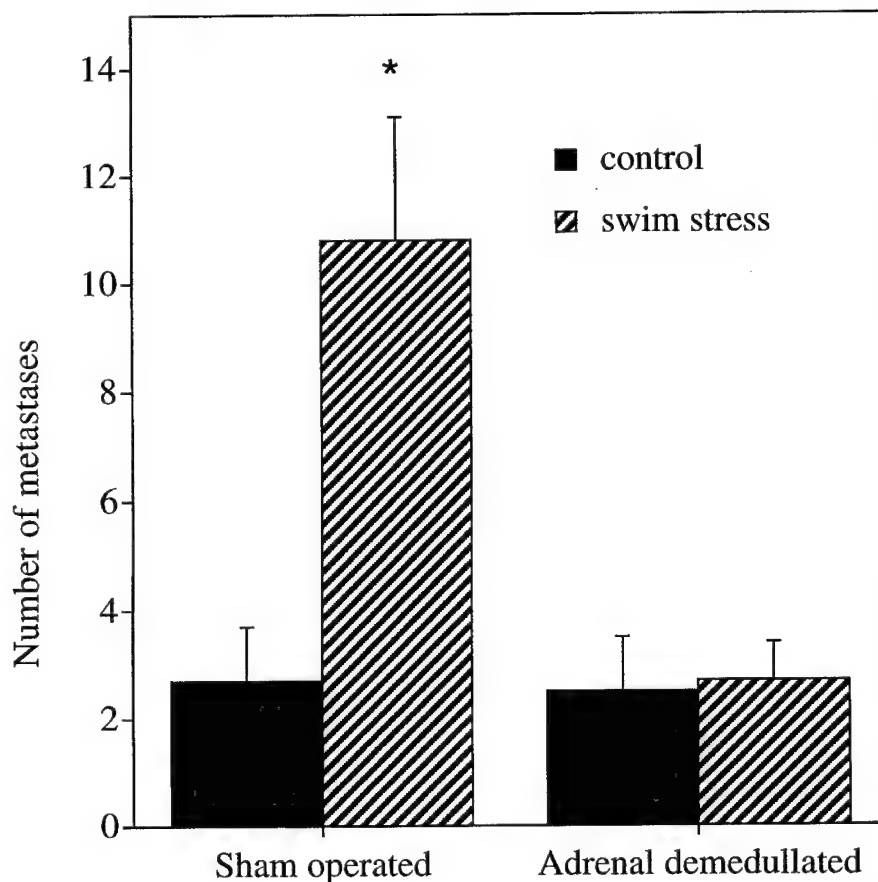


Figure 19.3 The effects of swim stress on the number of lung metastases in adrenal demedullated and sham operated rats. Error bars represent SEM. * — significantly different from control.

Taken together, these findings indicate a major role for adrenal catecholamines in mediating stress effects on the metastatic process of MADB106 tumor cells, and suggest a role for catecholamines in mediating the suppression of NK activity by stress.

Simulating the Effects of Stress by β -Adrenergic Activation: *In Vivo* Studies

To verify the role of β -adrenergic activation in mediating stress effects on an NK-sensitive metastatic process, we studied the *in vivo* response to a non selective β -adrenergic agonist, metaproterenol (MP). This drug is characterized by a long half life (several hours), providing prolonged activation of β receptors.

Metaproterenol (SC; 0.5, 1, 3 mg/kg) injected 1 hr before tumor inoculation, caused a dose-dependent increase in lung tumor retention, reaching an 8-fold effect (Figure 19.4). A significant increase in the number of lung metastases was also induced by MP (1 mg/kg) when metastases were counted three weeks after tumor injection. To support the suggestion that the increase in lung tumor retention is NK-mediated,

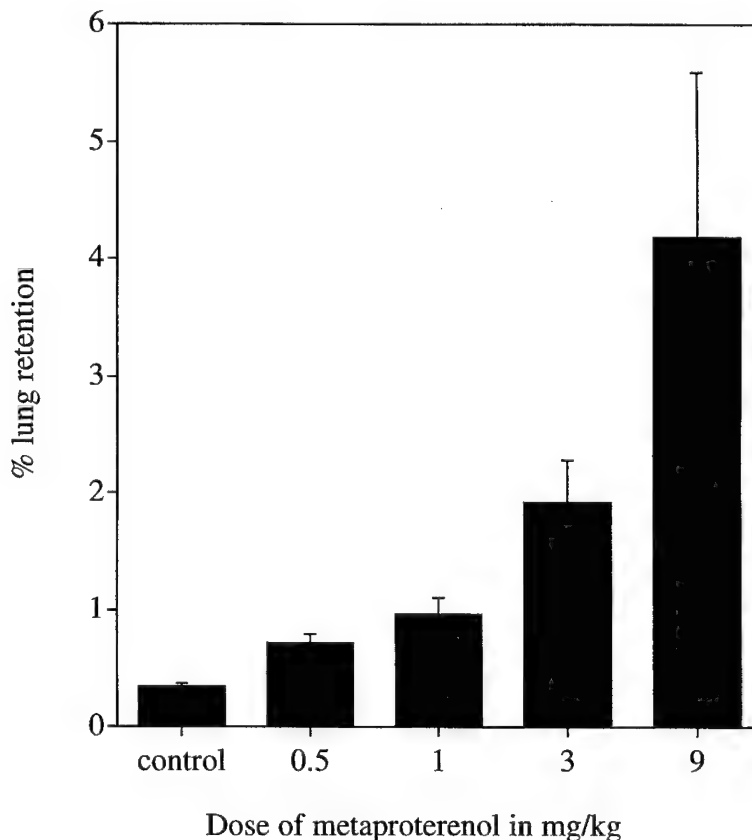


Figure 19.4 The effect of increasing doses of metaproterenol on lung tumor retention. Error bars represent SEM. All groups are significantly different from control.

we compared the effect of MP (0.5 mg/kg) between NK-depleted rats and control rats. Metaproterenol failed to increase tumor retention in the NK-depleted animals, but did increase tumor retention in the untreated rats, suggesting NK mediation of the effects of MP. The lack of an MP effect in NK-depleted rats is unlikely to be attributable to a ceiling effect, as other stress paradigms have been shown to increase tumor retention in such NK-depleted animals (see previous section, Figure 19.2).

Ex Vivo Studies of Adrenergic Stimulation

Ex vivo assessments of NK activity per ml blood were conducted 1 hr after IP injection of 0.1, 0.2, 0.4 mg/kg adrenaline or SC injection of 0.02, 0.06, 0.2, and 0.6 mg/kg MP. The middle dose of adrenaline used was the minimum needed to cause an increase in heart rate for at least 30 min following IP injection, and a similar effect was evident with 0.5 mg/kg of MP. Therefore, these doses appear to induce physiological levels of adrenergic activation.

Both adrenaline (Figure 19.5) and MP suppressed NK activity per ml blood in a dose-dependent manner, reaching a 50% suppression. Significant NK suppression was evident at all doses of both drugs except the lowest ones.

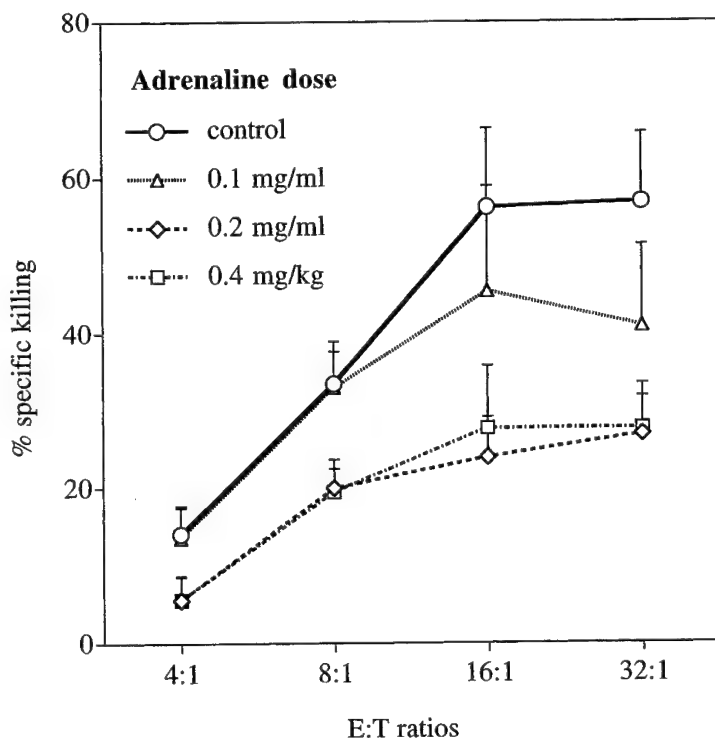


Figure 19.5 The effect of increasing doses of adrenaline on NK activity at 4 different effector to target ratios. Error bars represent SEM. 0.2 and 0.4 mg/kg are significantly different from control.

Redistribution of NK Cells

NK activity measured in a constant volume of blood, as was done in the above-mentioned studies, is dependent upon both the number of NK cells per ml blood and the activity per NK cell. Using flow cytometry we found that, at the time blood was drawn for the assessment of NK activity, MP did not change the number of NK cells (NKR-PI⁺ bright) per ml blood. Therefore, the suppression of NK activity, as measured per ml blood, indicates a reduction in NK activity per cell. Interestingly, MP did decrease the total number of leukocytes per ml blood, thus increasing the percentage of NK cells within the leukocyte population. Had we assessed NK activity per leukocyte, the increased ratio of NK cells within the leukocyte population might have overshadowed the NK suppressive effects of stress.

In Vitro Studies of Adrenergic Agonists

It is disputed whether adrenaline or β -adrenergic agonists suppress NK activity when applied *in vitro* (Hellstrand *et al.*, 1989). Using adrenaline in concentrations

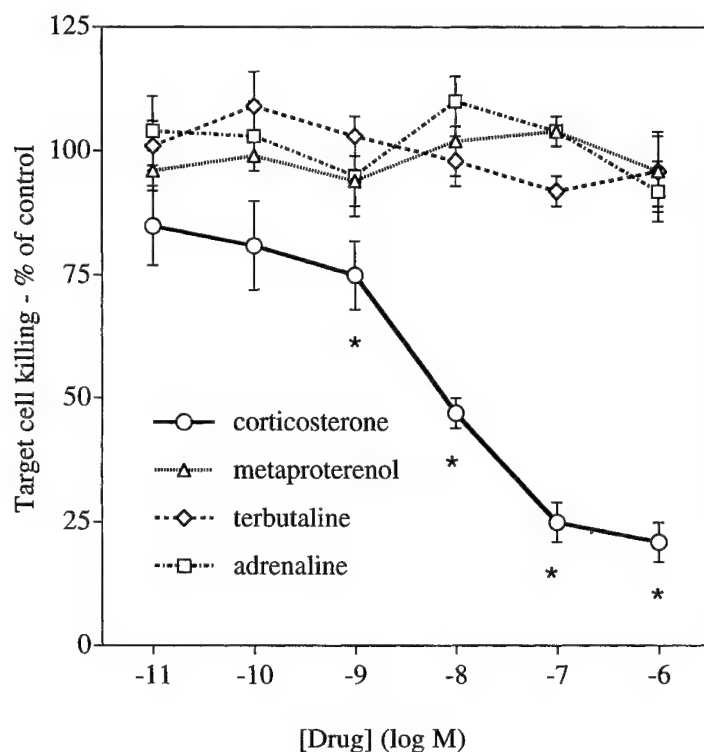


Figure 19.6 The *in vitro* effects of adrenaline, metaproterenol, terbutaline, and corticosterone on NK activity, expressed as percentage of control. Error bars represent SEM. * — significantly different from control.

ranging from low physiological to pharmacological (10^{-11} to 10^{-5} M), we were unable to demonstrate a decrease in NK activity (Figure 19.6).

The β -adrenergic agonists metaproterenol and terbutaline were similarly ineffective. In the same experiment we were able to demonstrate a marked reduction using physiological concentrations of corticosterone. Therefore, our current findings cannot suggest *direct* inactivation of NK cells by adrenaline.

Involvement of Central Adrenergic Mechanisms

Unlike adrenaline, MP crosses the blood-brain barrier (BBB); thus, its effects on NK activity may be centrally mediated. To test this hypothesis, we attempted to selectively block central and peripheral adrenergic receptors. To this end, we used two β -adrenergic antagonists, one that crosses the BBB (propranolol) and one that does not (nadolol). At low but equipotent doses (0.2 mg/kg; chosen for similar effects on heart rate), both β -blockers abolished the effect of MP on NK activity. Therefore, it appears that peripheral activation of β -adrenergic mechanisms underlie the NK suppressive effect of MP.

A ROLE FOR CORTICOSTEROIDS?

Corticosterone (CORT) is the major corticosteroid released during stress in rats and is known to suppress NK activity when applied *in vitro*. As demonstrated in our *in vitro* studies, this effect is induced at physiological concentrations (Figure 19.6), suggesting that corticosterone can mediate the effects of stress on NK activity and tumor metastasis. Nevertheless, several of our findings suggest that elevated physiological levels of CORT do not suppress NK activity *in vivo*, or that such suppression has no biological significance.

First, the stressed-induced increase in MADBI06 tumor metastasis was abolished by several methods of sympathetic blockade (e.g., β -adrenergic antagonist or adrenal demedullation), none of which are known to prevent the release of CORT. Furthermore, we conducted *in vivo* tumor retention studies following the administration of CORT (1, 3, 9 mg/kg; SC in oil; one hr prior to tumor). Corticosterone did not significantly affect lung tumor retention at doses verified to produce serum levels equal to or higher than stress levels. These studies suggest that elevated levels of CORT are not *sufficient* to suppress NK activity and to increase tumor metastasis. It could be suggested, though, that elevated CORT levels are a *necessary* condition for other factors to affect NK activity. We assessed this hypothesis with respect to the NK suppressive effects of MP that occurred in context of elevated CORT levels induced by this drug. Rats were pretreated with metyrapone, a CORT synthesis inhibitor, and the *ex vivo* levels of NK activity, as well as serum CORT levels, were assessed. Metyrapone abolished the MP-induced rise in CORT levels, but did not prevent the NK-suppressive effects of MP (Figure 19.7). Therefore, it is unlikely that the effect of metaproterenol on NK activity depends upon the rise in corticosterone levels.

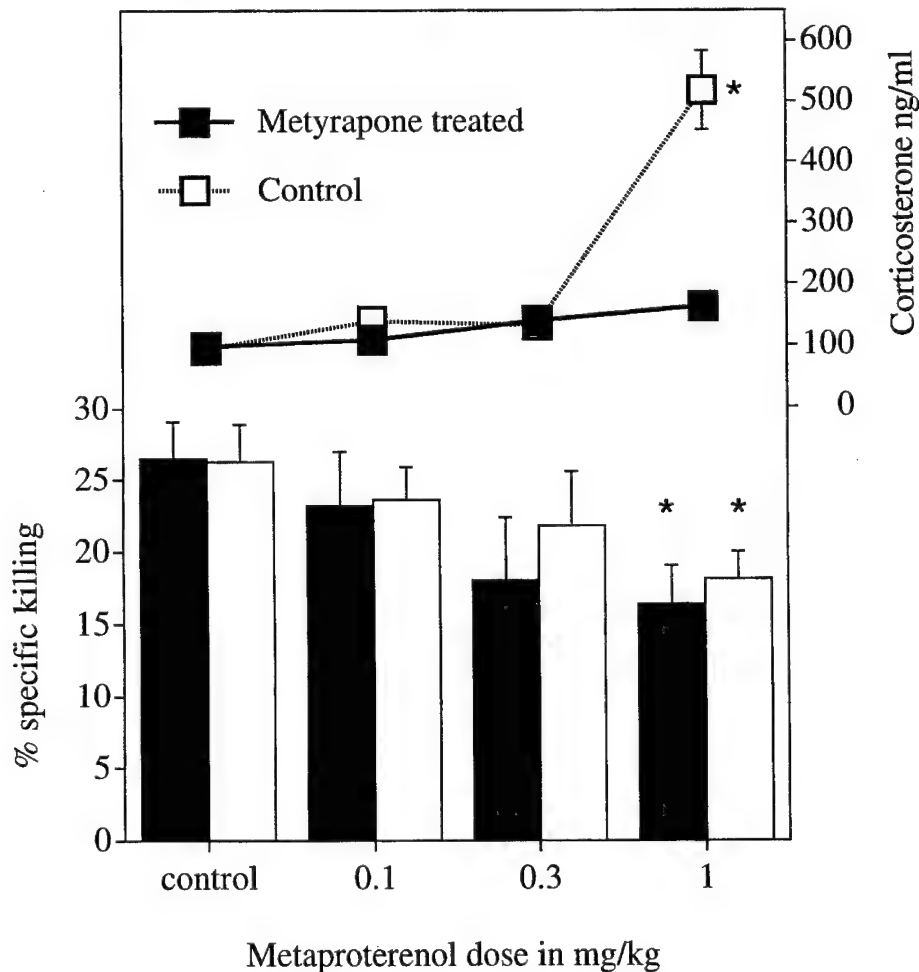


Figure 19.7 The Effects of different doses of metaproterenol in metyrapone-treated and control rats on NK activity (lower bar graph) and on serum corticosterone levels (upper line graph). Error bars represent standard errors of the mean. * — significantly different from control.

INTEGRATING REMARKS

The findings indicate a robust stress-induced suppression of cytotoxicity per NK cell and a simultaneous increase in host susceptibility to metastatic development of an NK-sensitive tumor. A causal relationship between the NK-suppressive and tumor-enhancing effects of stress is clearly indicated in some of the stress paradigms used. These findings attest to the biological significance of stress and of stress-induced NK suppression in experimental settings. The stress paradigms used are acute, and the tumor challenge is immense and well defined in time. Thus, the implications of these findings concern primarily similar clinical circumstances, such as the surgical

removal of a metastatic primary tumor. Nevertheless, in some chronic conditions such as alcoholism, suppression of NK activity is associated with a higher prevalence of infectious and malignant diseases, both of which have been shown to be controlled by NK activity. We have recently shown a causal relationship between acute ethanol intoxication, suppressed NK activity, and increased tumor metastasis (Ben-Eliyahu *et al.*, 1996). We have argued that such acute episodes are clinically relevant because there is a critical and short window of opportunity during which NK cells can prevent metastatic growth (Ben-Eliyahu *et al.*, 1996). Therefore, chronic suppression of NK activity by stress may be clinically relevant, as is acute suppression in conjunction with an existing chronic infection or metastatic process.

Whereas corticosteroids are known to be immune suppressive, our findings suggest that physiological levels of CORT *in vivo* do not suppress NK activity. On the other hand, *in vitro* CORT was shown by us and others to suppress NK activity. This discrepancy between *in vitro* and *in vivo* effects may be related to various differences in the hormonal and cellular milieu of the two environments. For example, the presence of corticosteroid-binding globulins (CBG) *in vivo* may markedly decrease levels of free CORT.

On the other hand, adrenaline and MP were ineffective *in vitro*, but markedly suppressed NK activity in *ex vivo* and *in vivo* settings. Adrenaline may induce a release of some NK-suppressive factor from a peripheral cell population absent *in vitro* (e.g., epithelial cells). Alternatively, a certain humoral factor or a biophysical condition necessary for the induction of adrenaline effects may be absent *in vitro*. Indeed, one group of researchers has repeatedly reported *in vitro* suppression of NK activity in human blood by adrenaline and β -adrenergic agonists (Hellstrand *et al.*, 1989).

Several human *ex vivo* studies suggested that infusion of adrenaline or stress-induced elevation of adrenaline levels *increases* NK activity (Tonnesen *et al.*, 1987; Kappel *et al.*, 1991); findings that seem incompatible with those reported herein and with the above *in vitro* findings using human blood (Hellstrand *et al.*, 1989). Apart from species differences, several factors may account for this apparent inconsistency. Most of the human *ex vivo* studies assessed NK activity during or shortly after adrenaline infusion, and assessed cytotoxicity per leukocyte or monocyte, rather than per NK cell. Because there is a sharp and short-lasting (approximately 1 hr) increase in the percentage of NK cells within the populations tested for NK activity, it was suggested that the observed increase is attributable to an increase in the number of NK cells (Kappel *et al.*, 1991). Our studies, on the other hand, assessed blood NK activity and tumor metastasis beginning 1 hr after stress or adrenaline administration, by the time number of NK cells had returned to baseline. Our findings in animals also indicate a decreased level of NK-mediated antimetastatic activity, which may depend on NK number and activity in immune compartments other than peripheral blood.

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20 Melatonin-Induced T-Helper Cell Opioid Cytokines with Anti-Stress and Hematopoietic Effects

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A bidirectional connection between the central nervous system (CNS) and the immune system is today widely recognized. The related research field is termed neuroimmunoendocrinology or psychoneuroimmunoendocrinology when emotions or psychological variables are considered in association with immune parameters. In this field, the pineal hormone melatonin has rapidly gained a prominent position as one of the most potent immunological active neurohormones. Melatonin can counteract stress or corticosteroid-induced immunodepression, protect mice against lethal viral infections and septic shock, synergize with interleukin-2 in cancer patients, reverse aging-associated immune defects, and rescue the blood forming system against the toxic action of cancer chemotherapeutic agents (Ben-Nathan *et al.*, 1995; Caroleo *et al.*, 1992; Champney and McMurray, 1991; Colombo *et al.*, 1992; Lissoni *et al.*, 1995; Maestroni, 1993, 1996; Maestroni *et al.*, 1994a,b; Morrey *et al.*, 1994; Pioli *et al.*, 1993). Enhanced production of T-helper cell cytokines upon melatonin binding to high affinity receptors (K_d : 346 ± 24 pM) seems to be the basic mechanism underlying these interesting effects (Maestroni, 1993, 1995). The antistress action of melatonin is neutralized either by T-helper cell depletion or by the specific opioid antagonist naltrexone. This suggested the involvement of T-cell derived opioid peptides (Maestroni, 1993; Maestroni and Conti, 1989, 1990).

As far as hematopoiesis is concerned, melatonin protects the blood-forming system of mice transplanted with Lewis lung carcinoma and treated with cyclophosphamide or etoposide (Maestroni *et al.*, 1994b). *In vitro*, melatonin rescues hematopoietic progenitors from apoptosis induced by etoposide or carboplatin, an effect that is neutralized by antigranulocyte/macrophage colony stimulating factor mAb. Consistently, melatonin, at both physiological and pharmacological concentrations, increases the number of granulocyte/macrophage colony-forming units (GM-CFU) when added directly in bone marrow cultures, but only in presence of suboptimal concentrations of colony stimulating factors (CSF) (Maestroni *et al.*, 1994b). These effects appeared to depend on a T-helper cell cytokine that is immunologically and

functionally indistinguishable from IL4 (Maestroni *et al.*, 1994a). Apparently, the IL4-like factor acts by triggering the production of CSF in adherent bone marrow stromal cells when they are activated by CSF and/or cancer chemotherapeutic compounds (Maestroni *et al.*, 1994a). However, further studies aimed at elucidating the melatonin-IL4 connection failed to confirm any effect of melatonin on IL4 mRNA expression or IL4 release (Maestroni, unpublished results). In a preliminary investigation we found that upon melatonin stimulation, either antigen-activated spleen mononuclear cells or nonadherent bone marrow cells secrete two similar opioid peptides (Maestroni *et al.*, 1995).

In the present study we investigated whether, in analogy with the immuno-enhancing and antistress action, the hematopoietic effects of melatonin are also mediated by the melatonin-induced opioids (MIO). We found that MIO indeed seem to mediate the hematopoietic effect of melatonin. In addition, we found indications that these substances, which resemble both IL4 and dynorphin B, might belong to a new family of opioid peptides and represent previously unrecognized hematopoietic cytokines.

EFFECT OF NALTREXONE ON THE HEMATOPOIETIC PROTECTION EXERTED BY MELATONIN

For a preliminary indication whether endogenous opioids are involved in the hematopoietic rescue exerted by melatonin in mice treated with a myelotoxic dose of cyclophosphamide, we evaluated the effect of naltrexone on blood cell counts and composition. We found that melatonin- and cyclophosphamide-treated mice had leukocyte counts significantly higher than those of mice treated with cyclophosphamide alone (Maestroni *et al.*, 1996). This effect appeared to depend on an increase of granulocytes. The contemporary administration of naltrexone counteracted part of the melatonin effect. This indicated that MIO mediate, at least in part, the hematopoietic rescue exerted by melatonin *in vivo*. To circumstantiate this result, we investigated whether naltrexone could also affect the hematopoietic effects of melatonin *in vitro*. The colony-stimulating activity of melatonin was completely neutralized by naltrexone. In contrast, the hematopoietic rescue *in vitro* was not significantly affected by the opioid antagonist while, as expected, anti-IL4 mAb was also effective (Maestroni *et al.*, 1996). Naltrexone or anti-IL4 mAb alone did not exert any significant influence on etoposide toxicity (Maestroni *et al.*, 1996).

SDS-PAGE AND IMMUNOBLOTTING CHARACTERIZATION OF MIO

Our previous work showed that melatonin induces the production of an IL4-like factor in bone marrow T-helper cells. Upon overnight incubation with melatonin (5 nM), the IL4-like activity was present in supernatants from nonadherent bone marrow cells, but not in supernatants from T-helper cells-depleted bone marrow cells (Maestroni *et al.*, 1994a). We thought to analyze these supernatants for the presence of MIO. After SDS-PAGE, an immunoblot procedure was performed

using a mAb that recognizes the amino terminal Tyr-Gly-Gly-Phe common opioid sequence. We found that nonadherent bone marrow cells incubated with melatonin secrete two proteins that are recognized by this antibody (Maestroni *et al.*, 1996). The apparent molecular weights of MIO are 15 and 67 kDa. To distinguish these molecules, we named MIO15 the lower molecular weight protein and MIO67 the larger one. Control cells incubated without melatonin released a much smaller amount of MIO (Maestroni *et al.*, 1996). When nonadherent bone marrow cells were depleted from T-helper cells, the immunoblotting was negative, confirming that MIO are produced by T-helper cells (Maestroni *et al.*, 1996). At this point, we wondered whether MIO also are recognized by anti-IL4 mAb, and this was indeed shown. We tried to further characterize MIO using mAbs directed against known opioid peptides. Anti-met-enkephalin, anti-leu-enkephalin, anti- β -endorphin, anti-dynorphin A Abs did not label MIO. In contrast anti-dynorphin B Abs could bind MIO67 but not MIO15.

In order to relate the hematopoietic effects of melatonin to MIO15 or MIO67, we decided to take advantage of their different molecular weights and separate them by gel filtration chromatography. After collecting and analyzing the various fractions, we found that MIO67 eluted in fraction 1 and MIO15 in fraction 4 (Maestroni *et al.*, 1996).

HEMATOPOIETIC EFFECT OF MIO

The gel filtration fractions containing MIO15 or MIO67 were tested for their colony-stimulating activity and capacity to rescue GM-CFU in bone marrow cells incubated with etoposide for 8 hours at 37°C. The fraction containing MIO15, but not that containing MIO67, exerted significant a colony-stimulating activity that was completely neutralized either by naltrexone or anti-IL4 mAb (Maestroni *et al.*, 1996). Conversely, both fractions rescued GM-CFU in bone marrow cells incubated with etoposide. In this case, naltrexone neutralized only the effect of MIO15 but not that of MIO67, while anti-IL4 mAb could abolish the effect of both (Maestroni *et al.*, 1996).

³H-NALOXONE BINDING

The presence of adherent bone marrow cells is needed for the colony-stimulating activity of the melatonin-induced IL4-like factor (Maestroni *et al.*, 1994a). The melatonin-induced factor that shows a colony-stimulating activity is MIO15 and its action is neutralized by both anti-IL4 mAb and naltrexone (Maestroni *et al.*, 1996). Naltrexone sensitivity indicates an opioid receptor-mediated effect. Therefore, we investigated whether adherent bone marrow cells express any specific binding site for ³H-naloxone. Isotherm saturation studies revealed that adherent bone marrow cells show a specific ³H-naloxone binding site. Nonlinear regression analysis gave the best fit for a single binding site with a K_d of 21.5 ± 9.8 nM and a B_{max} of 43 ± 14 fm/10⁶ cells, suggesting that the effect of MIO15 is mediated by opioid receptors present in adherent bone marrow cells. A scheme of the mechanism of action of MIO is depicted in Figure 20.1.

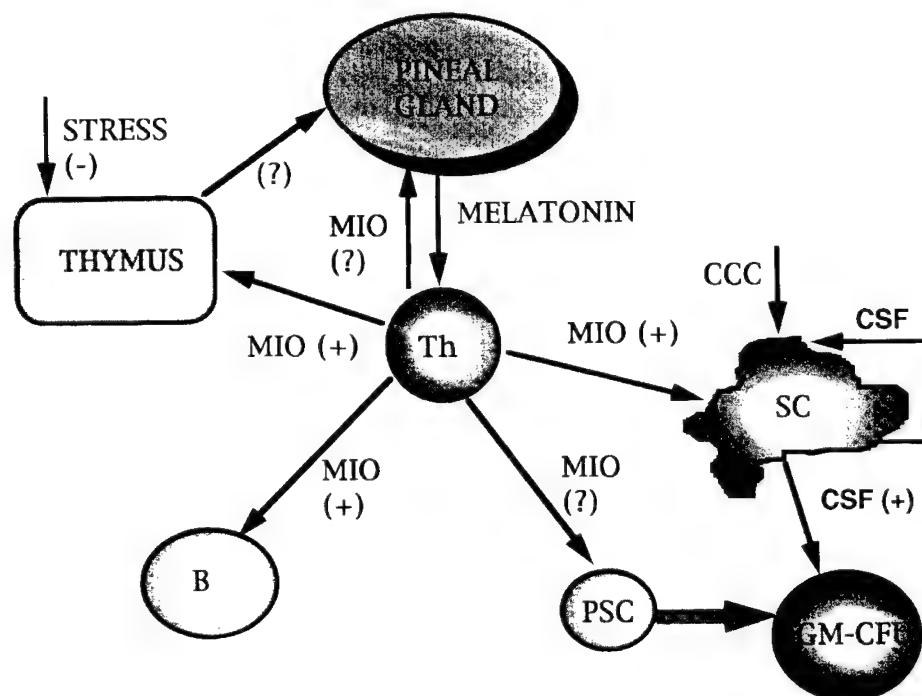


Figure 20.1 The melatonin-immuno-opioid network. Melatonin which is released by the pineal gland stimulates T-helper cells (Th) to release opioid peptides (MIO) which are recognized by anti-opioid and anti-IL4 antibodies. MIO might in turn inhibit pluripotent stem cells (PSC) and induce, via specific opioid receptors, activated stromal cells (SC) to release colony stimulating factors (CSF) which increases GM-CFU. This combined action rescues hematopoiesis from the toxic effect of cancer chemotherapy compounds (CCC). Activation of stromal cells may depend on the action of CCC or of CSF itself. On the other side, MIO may counteract the effect of stress (corticosteroids) on the thymus gland and on antibody production. The possible existence of feedbacks to the pineal gland from the thymus gland and T-helper cells is also hypothesized.

DISCUSSION

In the present study we show that the hematopoietic effects of melatonin are mediated by T-helper cell-derived cytokines with both opioid and IL-4-like features. This finding explains why we did not get any evidence for a direct melatonin-IL4 connection (unpublished results), despite the neutralizing effect of anti-IL4 mAb (Maestroni *et al.*, 1994a). Probably, this IL4 crossreactivity was revealed because anti-IL4 mAb was used at a rather high concentration (10 µg/ml). However, melatonin stimulates bone marrow T-helper cells to produce two proteins with an apparent molecular weight of 15 and 67 kDa that are recognized by both anticommon opioid sequence and anti-IL4 mAbs. These MIO seem to have different hematopoietic effects. MIO15 exerts a direct colony-stimulating activity and rescue GM-CFU from etoposide via a naltrexone-sensitive mechanism. The presence of a specific

³H-naloxone binding site in adherent stromal cells together with our preceding findings (Maestroni *et al.*, 1994a,b) suggests that MIO15 acts by triggering the production of CSF in adherent stromal cells via activation of opioid receptors. We are not aware of other studies reporting opioid receptors in bone marrow stromal cells. This finding might be relevant to our understanding of the role of the most widely studied molecule on the surface of leukemic cells, i.e., the common acute lymphoblastic leukemia antigen (CALLA, CD10), which has been recently found to be a neutral endopeptidase (E.C.3.4.24.11, enkephalinase; Delikat *et al.*, 1994; LeBien and McCormack, 1989). In contrast with MIO15, MIO67 does not enhance the number of GM-CFU, yet it exerts a significant protection against etoposide but its effect is naltrexone insensitive. This may explain why the hematopoietic rescue of melatonin *in vivo* was reduced partially by naltrexone while *in vitro* naltrexone was completely inactive. However, consonant with the immunoblotting characterization, the effects of both MIO15 and MIO67 are neutralized by anti-IL4 mAb. Although we have reported that bone marrow T-helper cells express a putative high affinity melatonin receptor (Maestroni, 1995), the mechanism by which melatonin stimulates MIO production is unknown. Immunoblotting studies indicated that MIO are released by T-helper cells while bone marrow T-helper cells incubated without melatonin could also release a small quantity of MIO. This may suggest that melatonin acts on posttranslational mechanisms although further studies are needed to clarify this important point.

In a preliminary report we showed that either antigen-activated spleen mononuclear cells or normal nonadherent bone marrow cells may secrete MIO. We reported a much lower molecular weight for MIO because of a technical mistake which occurred in the blotting procedure (Maestroni *et al.*, 1995). However, it seems now clear that either antigen-activated peripheral T-helper cells or bone marrow T-helper cells release the same molecules upon melatonin incubation. It is interesting to note that bone marrow T-helper cells do not seem to require any antigenic activation to respond to melatonin. This may reflect an inherent difference of bone marrow T-helper cells from peripheral T-helper cells and a physiological requirement for a sustained melatonin regulation of hematopoiesis.

As far as the nature of MIO is concerned, beside the anti-amino terminal Tyr-Gly-Gly-Phe common opioid sequence and anti-IL4 mAbs which label both molecules, only anti-dynorphin B Ab recognizes MIO67 but not MIO15. All the other anti-opioid Abs tested gave negative results. This suggests that MIO are related proteins with an amino terminal opioid sequence followed by a carboxy-terminal extension that share some sequence similarity with IL4 and dynorphin B. A free amino-terminal tyrosine in the common opioid sequence is essential for opioid receptor binding (Lord *et al.*, 1977) and this is consonant with our results. Interestingly, if one compare the aminoacidic sequence of IL4 with that of opioid precursor molecules, prodynorphin shows the highest similarity with IL4. It should also be considered that anti-leu-enkephalin Ab did not recognize MIO. This antibody shows only a 0.2% crossreactivity with Tyr-Gly-Gly-Phe-Leu-Arg, i.e., with the initial sequence of dynorphin. We also showed that known opioid peptides mimicked the antistress effect of melatonin and that the most potent was dynorphin 1-8 (Maestroni and Conti, 1989). Nevertheless, because of their size and unusual immunological

characterization, MIO cannot possibly be prodynorphin products. Therefore, we are tempted to speculate that MIO belong to a novel family of immunoderived, opioid-related proteins with immune and hematopoietic functions. Most recently, it has been reported the existence of a novel opioid peptide that resembles dynorphin (Meunier *et al.*, 1995) and MIO might be somewhat related to this peptide.

Acknowledgements

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V. Stress Effects: Implications to Human Conditions

21 Long-Term Behavioral Effects of Stress in Rats: Possible Animal Models of Post Traumatic Stress Disorder (PTSD)

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Different individuals will react differently, both in quality and in quantity, to the same stressful event. This variability, in humans, is demonstrated within the range described by an efficient coping with stressful events on the one hand and by the emergence of psychiatric disorders such as anxiety, depression or PTSD on the other. One of the major parameters that may affect the coping response is the extent of previous exposure to stress conditions. The exact nature of these conditions, such as their predictability, controllability, length of exposure, or stimuli involved are a matter of debate as can be attested to by the myriad of animal models described.

The study of the long-lasting effects of acute or chronic stress in humans will clearly benefit from the development of the appropriate animal model. Such models can be used to further understand processes associated with stress related disorders as well as the behavioral or pharmacological manipulations that will help alter their course. Although the ultimate animal model should present all aspects of a specific disease, the variety of stress related disorders will probably be best modeled with a number of experimental procedures each presenting one or more aspects of long-term effects of stress.

The increased interest in PTSD (Foa *et al.*, 1992; Yehuda and Antelman, 1993) highlights the difficulties associated with the treatment of this long-term disorder. Appropriate animal models may offer insight into its development and may point to a critical period for possible treatments. The present work is an attempt to review some animal models of stress exposure and evaluate them in relation to PTSD symptoms.

Psychiatric diagnosis of PTSD is based on the DSM-III-R (*Diagnostic and Statistical Manual of Mental Disorders*, American Psychiatric Association, 1987) and is described in detail elsewhere in this book (Shalev and Sahar). The symptoms emphasize emotional disturbance and are grouped under reexperiencing, avoidance, and arousal. Symptoms of PTSD were described and analyzed by Foa *et al.* (1992) in an attempt to define processes of the disorder that may help direct animal research

and generate animal models. In this extensive review, the nature of the stressors producing PTSD were specifically addressed. The authors suggested that in addition to the stressor being "markedly distressing to almost anyone" (DSM-III-R) and perceived as a threat to one's life, it must also be experienced as uncontrollable or unpredictable (Foa *et al.*, 1992).

The effects of controllability and predictability of stress was extensively studied in both animals and humans. The various paradigms were termed "learned helplessness", "experimental neurosis" or "behavioral despair" (DeWald and Thomas, 1976; Maier, 1991; Porsolt *et al.*, 1978; Seligman, 1975). These paradigms repeatedly demonstrated behavioral deterioration following stress exposure. In a learned helplessness paradigm, rats pre-exposed to a long series of inescapable and uncontrollable footshocks failed to learn subsequent response to specific escape or avoidance tasks (Overmier and Seligman, 1967; Maier *et al.*, 1973; Warren *et al.*, 1991). The cognitive impairment, often associated with reduced motility (Woodmansee *et al.*, 1993), was explained by an acquired helplessness, and was suggested as a model for some aspects of human depression (Seligman, 1975). Emotional aspects of this paradigm included generalized fear (Maier, 1990) and an increase in selective attention to exteroceptive cues (Warren *et al.*, 1991). Thus, some aspect of PTSD may be present in the paradigm. However, these experiments failed to model the long-term debilitating effects seen in PTSD, since learned helplessness was demonstrated for only a short period (2-3 days) following the stress exposure.

Exposure to intermittent footshocks induced long-term decrease in activity (Van Dijken *et al.*, 1992). This was detected for as long as 42 days following exposure (Maier *et al.*, 1990) and may be independent of escapability (Woodmansee *et al.*, 1993). Behavioral effects induced by this relatively short session of inescapable shocks were examined as a model of anxiety. Thus, long-lasting hyper-responsiveness was detected in the stress exposed rats in a forced swim test and in a sudden noise change test. The stressed rats also showed a general decrease in locomotion and increased immobility that were detected from 1 to 21 days after stress but were not present if the rats were tested immediately after the stress session (Van Dijken *et al.*, 1992a,b; Prince and Anisman 1984). Increase in the number of shocks during the stress session may result in the disappearance of these long-lasting behavioral changes (Van Dijken *et al.*, 1992b,c). Indeed, if the stress sessions were repeated for 10 days, activity returned to baseline levels, probably as a result of the protective process of stress adaptation (Ohi *et al.*, 1989; Ottenweller *et al.*, 1992).

In an attempt to attenuate stress adaptation, Katz and collaborators suggested an animal model based on chronic, intermittent exposure to unpredictable stress. They exposed rats to various stressors for 21 days and 2-3 days later tested the animals for their reaction to 1 hr of noise stress. The stressed animals showed moderately depressed open field activity, and failed to over-react to the noise stress, as compared to non-stressed controls (Katz *et al.*, 1981). Although their behavior may parallel the stress-induced increased immobility reported above, the decreased reactivity to the noise stress was interpreted as a depressive reaction. Since a prior history of stress was believed to be a possible precipitant of certain forms of depression in humans, this model was extensively tested as an animal model of hedonic deficits (Katz, 1982; Katz and Baldrighi, 1982; Katz and Sibel, 1982a,b). Anhedonia was also demonstrated

following 3 to 6 weeks of variable, unpredictable stress with relatively mild stressors (Papp *et al.*, 1991). These stressed animals failed to increase their consumption of sucrose solution (Katz, 1982; Willner *et al.*, 1987; but see also Matthews *et al.*, 1995), and showed little preference for food pellets, sweet solutions or amphetamine in a place preference conditioning paradigm (Papp *et al.*, 1991). Similarly, stress exposure altered reward properties as assessed by behavioral changes in intracranial self stimulation at dopaminergic sites (Moreau *et al.*, 1992; Zacharko and Anisman, 1991).

The model of repeated variable stress was analyzed in terms of face and construct validity as a model for human depression (Willner, 1984). However, the relation between stress and depression was questioned for various reasons: the passivity seen in the behavior of the stressed rats is not common to all types of human depression, the relation between stress and human depression is not clear, and causal relations between stress and depression are not easily demonstrated (Willner, 1984). In contrast, the relation between stressful events and PTSD is clear, as stress is part of the definition of this disorder. The stress may be short and "traumatic" or culminate over a long period of life (Shalev and Sahar, in this book). A history of stress exposure may result in a susceptibility to develop PTSD following a traumatic event. It may also accelerate the development of the disorder following repeated reexperiencing of the trauma. Thus, the variable unpredictable stress model was extended and re-examined here as a possible model for PTSD.

Long-lasting behavioral effects were examined in male Sprague-Dawley rats after 6 weeks exposure to variable unpredictable stressors. The analysis focused on effects lasting for months following the exposure. Stressors were administered at all hours and included footshocks, re-exposure to the shock environment, immobilization, isolation, shaking, partial water deprivation, blood sampling, cold exposure, and a day/night shift. The order of stressors presentation, their duration and the time of day they were applied were random within and between experiments.

The animals were not tested during the stress exposure period. At the end of the six weeks of stress exposure, the animals were repeatedly tested in an open field for a 30 min session at various times following exposure. The movements of the animals in the open field were monitored via a computerized visual tracking system (HVS, England). The most striking behavioral effect seen was a significant increase in open field activity in the stressed animals compared to their non-stressed controls. With repeated tests habituation to the test environment was seen in both stressed and non-stressed rats, but the increased mobility in the stressed rats was seen even at ten months following termination of the stress period (Figure 21.1). The increased mobility was not due to an increase in speed, since the stressed rats showed a parallel increase in the percent of time they were active in the field (Figure 21.1). Thus, previous exposure to the stress induced 'restlessness' that may represent higher anxiety level in the mildly stressful environment of the open field. The possibility that the effects dissipate with time, and not because of repeated testing, was ruled out in a comparison made 10 months following stress exposure. In two separate experiments, stressed and non-stressed animals were repeatedly tested either 3 or 6 times in the open field. Results showed that the extent of the group differences depended on the number of exposures to the same environment (Figure 21.2). Thus, the

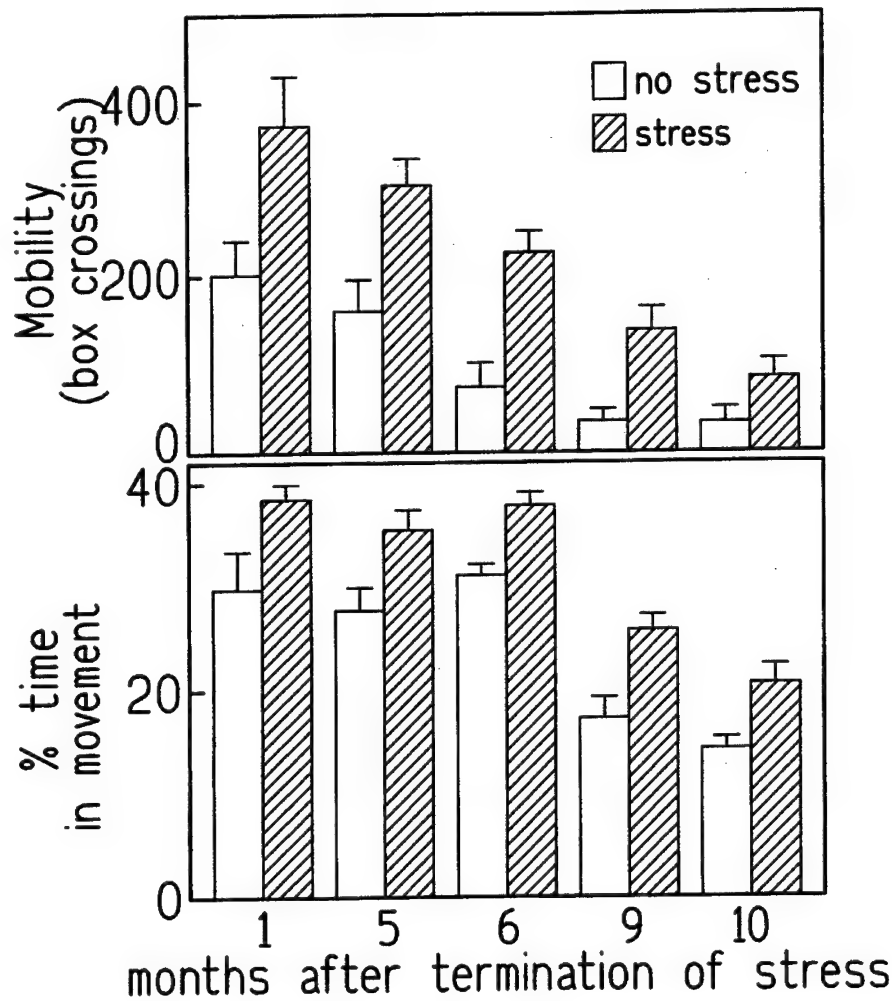


Figure 21.1 Behavior in the open field. Rats were exposed to 6 weeks of variable, unpredictable stress and were tested repeatedly in 30 min sessions, 1 to 10 months following the termination of stress. Mobility: number of movements across lines dividing the area to 25 boxes, 20 × 20 cm each. (mean ± SEM).

stress-induced behavioral changes did not diminish with the passage of time and were present at least 10 months following the termination of stress.

All the open field tests were carried out in 30 min sessions. Since similar experiments commonly use shorter session time, the 30 min sessions described in Figure 21.1 were re-analyzed in 10 min segments (Figure 21.3). The increased mobility following stress exposure can be clearly seen throughout the session. Although within session habituation (decrease mobility) was seen in both stressed and non-stressed rats, the 'saving' from session to session was partially impaired in the stressed rats (e.g. Figure 21.3, comparison of performance in the last segment of the 6th month to the first segment

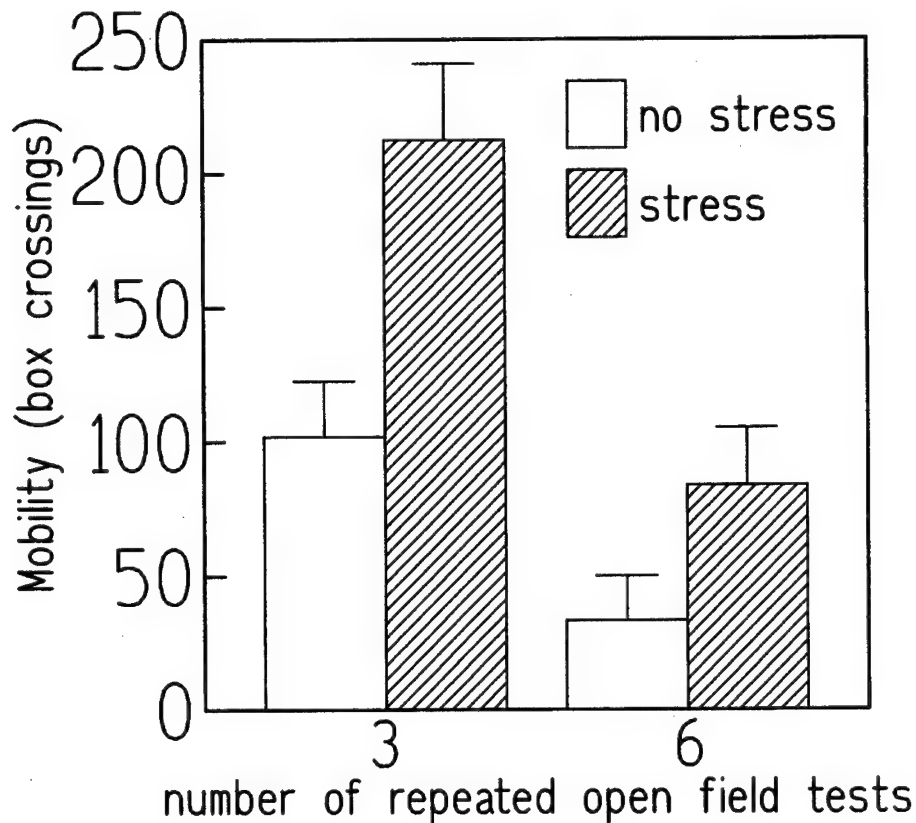


Figure 21.2 Behavior in the open field 10 months following the termination of stress. In two separate experiments, stressed and non-stressed rats were repeatedly tested either 3 or 6 times (in 30 min sessions) in the open field prior to the present test. See Figure 21.1 for more details.

of the 9th month following stress termination). The data suggest the possibility of impaired memory function, as was previously reported after prolonged exposure to stress, corticosterone or years long PTSD (Sapolsky, 1996a; Bremner *et al.*, 1993). Testing the stressed rats in a working memory paradigm in the Morris water maze (Morris, 1984) revealed no impairment in acquisition of the task compared to the non-stressed controls. However, water maze testing took place during the first or second week following termination of stress, which may be too early for the detection of memory deterioration (Sapolsky, 1996b).

The stressed exposed rats were further tested for possible hedonic deficiencies. Results showed that the increased activity was not accompanied by weight loss or a decreased preference to sweet water solution, described above in animal models of depression (Katz, 1982; Rapp *et al.*, 1991). In a forced swim test, the stressed animals were more active than the non-stressed controls. This is in accord with the previously reported shock-induced decreased immobility in this test, a response suggested to reflect arousal, panic or anxiety (Prince and Anisman, 1984). Thus,

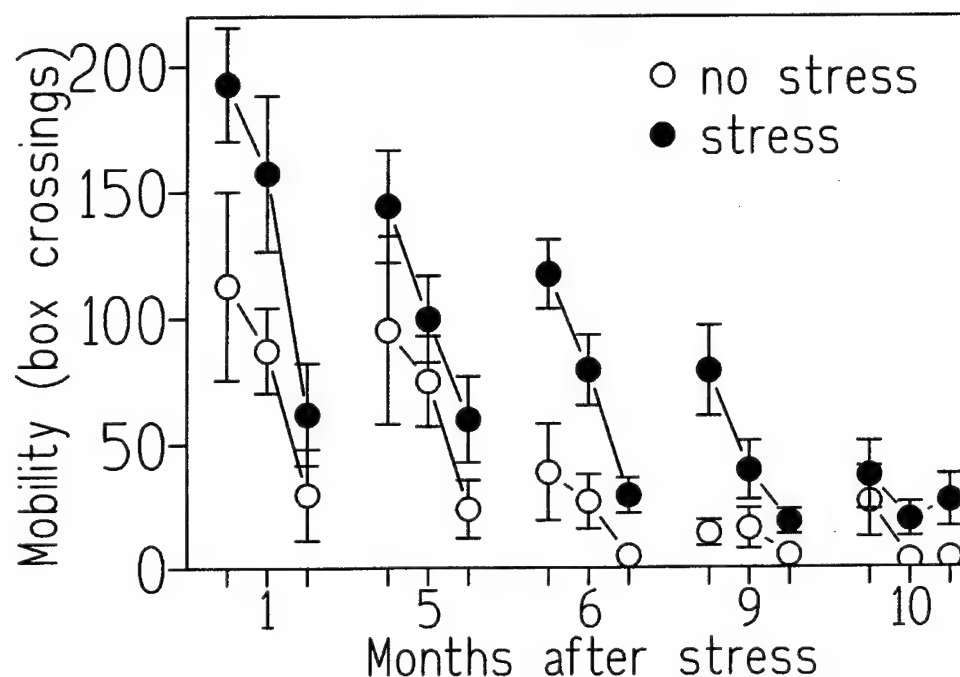


Figure 21.3 Behavior in the open field. Part of the data in Figure 21.1 is presented in 10 min segments. See Figure 21.1 for more details.

exposure to intermittent, variable, unpredictable and extensive stress period failed to alter hedonic properties but induced behavioral arousal that lasted for months following termination of stress and may represent similar increase in arousal seen in PTSD.

The contingency between the duration and the variability of stress exposure and the ensued long-lasting behavioral changes is not yet clear. When the effects of some of the stressors were tested separately and for a short exposure period (1 session/day for 2 successive days, rats tested 2 days later), a decreased open field activity was seen (Figure 21.4). This decrease in exploratory behavior is similar to that described as an animal model of depression following either variable or single modality stress exposure. The reasons for the opposing behavioral outcome is not clear. There are major differences in the paradigms and in the schedules of testing that may account for some of the discrepancies. A complicated aspect is the intensities of the stressors used; for example, in the present experiments, only one footshock session was administered per 6 weeks of stress exposure, and the session was comprised of only 2 shocks delivered at least 10 min apart. Others used repeated footshock sessions, 10 to 60 footshocks per session and shocks were delivered, on the average, every 90s (Katz, 1982; Van Dijken *et al.*, 1992). Exposure to this learned helplessness-induced sessions may have contributed to the general suppression of responses seen in these animal models of depression. However, stress intensities *per se* could not explain the

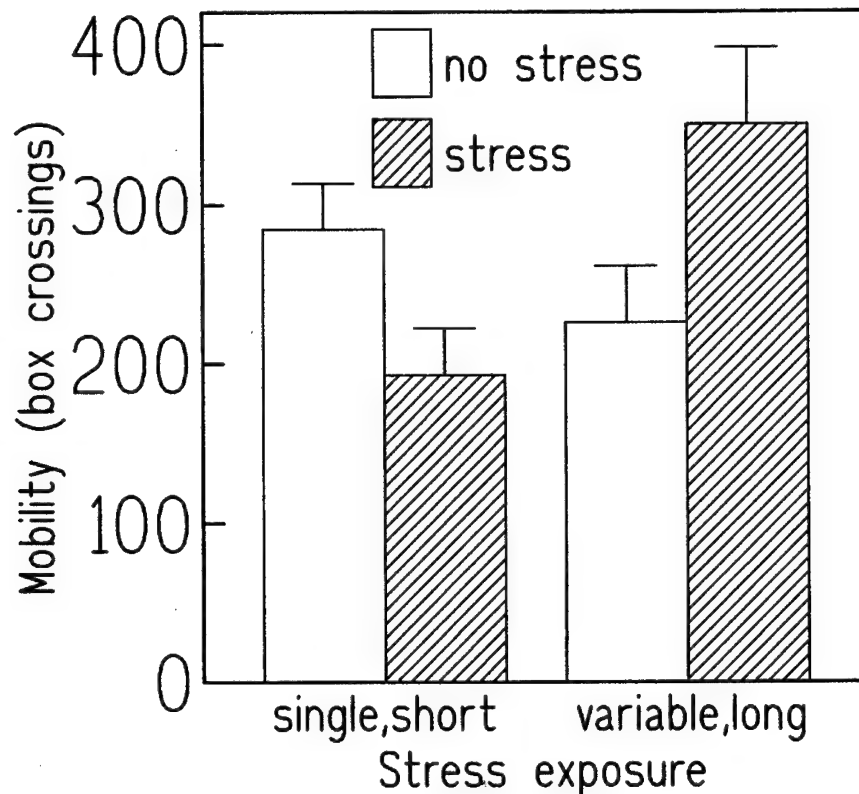


Figure 21.4 Comparison between the effects of short stress exposure (2 foot shocks/day for 2 successive days, rats tested 2 days later. Similar data were obtained following shaking stress or restraint stress) and the effect of 6 weeks of variable unpredictable stress exposure on open field behavior. See Figure 21.1 for more details.

different outcome, since not all stressors in the present experiments were as short lasting or as mild (e.g. 2 hr immobilization, or 4 hr at 4°C).

Extensive experience with stress, uncontrollable and inescapable, resulted in long-term behavioral hyper-arousal. These long-lasting behavioral effects may represent a 'breakdown' in the stress habituation processes that are not easily restored. This may parallel a possible course of events where by previous exposure to stress contributes to the 'breakdown' described as PTSD.

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22 Neurobiology of the Post Traumatic Stress Disorder[☆]

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Post traumatic stress disorder (PTSD) is a chronic and pervasive anxiety disorder that follows exposure to extreme stress. Descriptions of combat-related disorders in the medical literature can be found as far back as 17th century (Rosen, 1975). Yet, it is only in 1980 that the diagnostic criteria for PTSD have been delineated and formally introduced into an official classification of mental disorders (APA, 1980). Three clusters of symptoms characterize PTSD, including (a) distressing recurrence of the traumatic experience, (b) avoidance of cues reminiscent of the traumatic event and numbing of general responsiveness, and (c) hyperarousal (Table 22.1). In its current definition (APA, 1994), PTSD may result from a variety of events, including military and civilian traumata as well as natural disasters.

Symptoms resembling those of PTSD are expressed by the majority of trauma survivors during the days that follow the trauma. These early symptoms, however, abate with time, leaving some survivors (e.g., 15% of Vietnam combat veterans) with chronic PTSD. Recovery from chronic PTSD is rare and the effect of treatment (pharmacological or psychological) is limited. Chronic PTSD is often associated with other psychiatric disorders, such as depression or substance abuse.

Whilst traumatic exposure is the visible trigger of PTSD, variables that precede the trauma contribute substantially to the causation of the disorder. Epidemiological studies have identified numerous factors that increase the likelihood of developing PTSD upon exposure to a trauma (for review, see Shalev, 1996). These factors include early separation from one's parents, mental illness prior to the trauma, personality traits such as introversion, and lifetime exposure to violence. From a biological perspective, inherited vulnerability and attrition of CNS capability to respond to stress through repeated traumatization, are such "prior" risk factors.

Dimensions of the stressful event that increase the likelihood for developing PTSD are the intensity of the event, its duration, its predictability and its controllability. Postevent variables such as social support and secondary stressors (e.g., pain following injury, relocation following a disaster) affect the likelihood for developing

Table 22.1 DSM IV diagnostic for post traumatic stress disorder (PTSD)

<i>A. The person has been exposed to a traumatic event</i>
(1) the person experienced, witnessed, or was confronted with an event or events that involved actual or threatened death or serious injury, or a threat to the physical integrity of self or others
(2) the person's response involved intense fear, helplessness, or horror
<i>B. The traumatic event is persistently reexperienced (at least one symptom)</i>
(1) recurrent and intrusive distressing recollections of the event
(2) recurrent distressing dreams
(3) acting or feeling as if the traumatic event were recurring
(4) intense psychological distress at exposure to internal or external cues that symbolize or resemble an aspect of the event
(5) physiological reactivity on exposure to internal or external cues that symbolize or resemble an aspect of the event
<i>C. Avoidance of stimuli associated with the trauma and numbing of general responsiveness (at least three symptoms)</i>
(1) efforts to avoid thoughts, feelings, or conversations associated with the trauma
(2) efforts to avoid activities, places, or people that arouse recollections of the trauma
(3) inability to recall an important aspect of the trauma
(4) markedly diminished interest or participation in significant activities
(5) feeling of detachment or estrangement from others
(6) restricted range of affect
(7) sense of a foreshortened future
<i>D. Persistent symptoms of increased arousal (at least two symptoms)</i>
(1) difficulty falling or staying asleep
(2) irritability or outbursts of anger
(3) difficulty concentrating
(4) hypervigilance
(5) exaggerated startle response
<i>E. Duration of the disturbance is more than 1 month</i>
<i>F. Presence of significant distress or impairment</i>

PTSD as well. Studies have also assessed the extent to which PTSD can be predicted from the immediate response to the trauma, and there is a general agreement that the intensity of the early response is a sensitive, yet nonspecific predictor of PTSD. Indeed, survivors who do not express extreme behavioral disturbances during the event (e.g., do not "break down" during combat) may still develop PTSD later. Hence, the extent to which knowledge about acute responses to stress is relevant to PTSD is a matter of debate.

Given the complex etiology, chronic course, and poor prognosis of PTSD, the primary goals of biological research for the disorder seem to be: (a) to explain the process by which some trauma survivors develop PTSD while most others recover; (b) to evaluate the biological mechanisms that underlay PTSD and assess their specificity with regard to other mental disorders; and (c) to generate hypotheses that might lead to effective treatment. In evaluating the neurobiology of PTSD one must also be aware, however, that: PTSD has only recently become the object of systematic research (research in depression and schizophrenia has started in the 19th century); PTSD is a generic response to all types of trauma; and that PTSD is a truly postexposure disorder, rather than a stress response. The following text outlines the biological findings in PTSD, examines the related theories, and discusses future research and clinical directions. The following areas are addressed: genetic

epidemiology, neuroendocrinology, psychophysiology, memory studies, and brain-imaging studies.

GENETIC EPIDEMIOLOGY

The contribution of heredity to the occurrence of PTSD has been explored by family and twin studies. Family studies of Vietnam veterans have shown higher prevalence of alcoholism, depression, and anxiety disorders in family members of PTSD probands (Davidson, 1985, 1989). However, a more recent study of rape survivors failed to show an increase in the prevalence of anxiety disorders amongst first-degree relatives of survivors with PTSD (Davidson, in press). Depression in family members was linked to the presence of depression in PTSD and was not significantly higher amongst relatives of PTSD probands who did not suffer from concurrent depression.

Using the large Vietnam Twin Registry, True and colleagues (1993) evaluated the relative contribution of heredity and of combat exposure to the occurrence of PTSD symptoms, comparing monozygotic to dizygotic twins with and without combat exposure. They found that genetic factors contribute to the occurrence of PTSD and its symptoms above and beyond the effect of combat exposure. Intrusive PTSD symptoms were better accounted for by combat exposure, while genetic factors determined up to 34% of the variance in avoidance and hyperarousal. Given that PTSD develops after trauma, it is possible that the relevant genetic traits are only expressed after exposure.

NEUROENDOCRINOLOGY

Neuroendocrinological studies have explored the classical "stress hormones," catecholamines, and the hypothalamic-pituitary-adrenal (HPA) axis. For each system, the baseline activity has been explored, as well as the effects of behavioral and biochemical challenge.

Central and Peripheral Catecholamines

Increased heart rate (HR), blood pressure (BP) and sympathetic activation, which regularly accompany acute stress responses, are mediated by the noradrenergic brain systems and the sympathetic nervous system. Within the CNS the locus coeruleus (LC) is the site of the majority of the noradrenergic neurons in the brain and projects to limbic structures involved in learning and memory (e.g., hippocampus, hypothalamus, amygdala) and to the prefrontal cortex. Through its projections, the LC is involved in orienting and alarm responses, phasic (panic) anxiety, memory consolidation and emotional activation. Acute stress results in a release of norepinephrine in the hippocampus, hypothalamus, and other brain areas. Chronic stress is associated with long-term changes in noradrenergic brain systems, such as sensitization and hyperresponsiveness to further challenge.

Basal sympathetic activity

Symptoms "resembling the effects of an injection of adrenaline" have been described amongst impaired combat veterans of World Wars I and II. Findings of basal sympathetic activity in PTSD are, however, diverse (see Southwick, 1993). Elevated resting HR has been reported in PTSD patients by some authors, typically while they were expecting a stressful task, such as awaiting a medical examination in an emergency room or waiting to be exposed to cues reminding the trauma (McFall and Murburg, 1994). Other studies failed to show a difference in resting HR between unchallenged PTSD patients and controls. A recent study (Shalev *et al.*, in press) has shown that trauma survivors who later develop PTSD showed significantly higher HR upon admission to a hospital following trauma (96.4 ± 13.9 vs 83.4 ± 10.8 in trauma survivors who did not develop PTSD). The difference between the groups disappeared one month after trauma.

Studies of urinary excretion and plasma levels and of catecholamines yielded controversial findings, some showing higher and others showing normal levels (Table 22.2). Moreover, elevated urinary excretion of epinephrine was found in survivors of stressful events regardless of the occurrence of PTSD (Baum *et al.*, 1983), and may, therefore, be related to exposure, or to chronic stress. Decreased α_2 adrenergic receptor binding sites on peripheral platelets was found in PTSD and in survivors of child trauma (Perry *et al.*, 1987). A possible explanation of the discrepancy is the lack of adequate control of physical and mental activity. Indeed, when blood samples were taken from resting PTSD patients, 30 minutes after catheterization, there were no differences between PTSD and control patients (McFall *et al.*, 1990). Hence, the observed difference in 24-hour excretion of catecholamines may confound the effect of phasic activation (i.e., adrenergic hyperresponsiveness) with those of baseline activity.

Responses to behavioral challenge

Contrasting with the above, studies of behavioral challenge (e.g., exposure to trauma-related stimuli) have consistently demonstrated an increase in HR, skin conductance, plasma epinephrine, and plasma norepinephrine in PTSD (reviewed by Shalev and Rogel-Fuchs, 1993).

Responses to biological challenge

Lactate infusion, which induces panic attacks in panic disorder patients, provokes panic attacks and flashbacks in PTSD patients. Similarly, yohimbine, an α_2 -adrenergic receptor antagonist, which activates NE activity by inhibiting the α_2 autoreceptor, produces panic attacks in 70% and flashbacks in 40% of PTSD patients (Table 22.2). Yohimbine also enhances the responses to auditory startle in PTSD. However, anxiety and flashbacks can also be provoked in PTSD patients by the 5-HT agonist MCPP. Interestingly, PTSD patients who so react to MCPP do not respond to yohimbine challenge and vice versa (Krystal, 1996), suggesting a degree of biological heterogeneity in PTSD.

Table 22.2 Catecholamines in PTSD

Author	Outcome measure	Study groups	Results
<i>Steady state</i>			
Kosten <i>et al.</i> , 1987	Urinary NE, EPI	9 PTSD; 8 MDD; 8 BP, 12 P-SCH; 7 SCH	Higher Urinary NE in PTSD, However — lower than Bipolar
Yehuda <i>et al.</i> , 1992	Urinary Dopamine, NE, EPI	22 VnVs w PTSD 16 normal controls	Higher excretion of all 3 catecholamines. Dopamine and norepinephrine correlate w/ severity of PTSD
McFall <i>et al.</i> , 1992	plasma NE, EPI, HR, BP	11 PTSD and 11 nl. controls	No significant difference between PTSD and controls in tonic sympathetic activity
Davidson <i>et al.</i> , 1985	Platelet MAO activity	23 PTSD patients and 19 nl. controls	Lower MAO activity in PTSD w/ history of alcohol abuse
Perry <i>et al.</i> , 1987	Platelets Alpha ₂ Adr. Binding and affinity	Several studies — veterans	Down regulation of peripheral alpha ₂ receptors. Might be due to chronic distress
<i>Provocation tests</i>			
McFall <i>et al.</i> , 1990	HR BP and Plasma catecholamines responses to combat stress films	10 VnVs w/ PTSD and 11 nl. controls	Higher HR BP EPI response in PTSD
Blanchard <i>et al.</i> , 1991	Plasma NE after exposure to combat auditory stimuli	15 VnVs w/ PTSD and 6 nl. veterans	30% rise in plasma NE in PTSD; no change in controls
Southwick <i>et al.</i> , 1993	Yohimbine infusion	20 PTSD and 18 nl. controls	Panic attacks in 14 patients, flashbacks in 8 patients. Higher MHPG response in PTSD

Abbreviations: NE = Norepinephrine; EPI = Epinephrine; HR = Heart rate; BP = Blood pressure; AO = Monoamine oxidase; VnVs = Vietnam Veterans; PTSD = Post traumatic stress disorder; MDD = Major Depression Disorder; SCH = Schizophrenics; P-SCH = Paranoid Schizophrenics; BP = Bipolar Disorder; nl. = Normal.

Combined with steady-state studies, the resulting evidence suggests that a normal or even lower baseline noradrenergic activity is coupled in PTSD with increased phasic activation upon exposure to a large variety of stimuli. Normal life stressors (e.g., loud noises, quarrel, anxious expectation) may trigger excessive adrenergic responses in PTSD patients, which they may attempt to avoid by reducing involvement in daily activities.

HPA Axis

The HPA axis plays a major role in the stress response. Acute stress results in an activation of corticotropin releasing factor (CRF)-secreting cells in the paraventricular nucleus (PVN), ensuing activation of adrenocorticotrophic hormone (ACTH)-secreting cells in the pituitary, and an increase in circulating cortisol. Cortisol affects many stress-related responses (e.g., gluconeogenesis) and containment of inflammatory responses. Cortisol also inhibits hypothalamic and pituitary secretion of CRF and ACTH, thereby deactivating the HPA axis. The inhibitory effect of circulating cortisol extends to central noradrenergic system. CRF itself mediates the response to stress in several brain areas such as the central nucleus of the amygdala, the hippocampus, and the prefrontal cortex. CRF also increases locus coeruleus firing and enhances startle response. Conversely, LC activity enhances CRF secretion at the PVN. Beyond its hormonal effect, cortisol induces a series of intracellular events, which, under extreme conditions, may result in cellular damage — particularly to the hippocampus and related structures (McEwan, 1995).

Basal HPA activity in PTSD

Despite its status as a “stress disorder,” studies have repeatedly indicated that 24-hour cortisol excretion in persons with PTSD is lower than that observed in depressed and normal individuals (Table 22.3). More lymphocyte glucocorticoid receptors (GCRs) were found in PTSD patients; moreover, the number of GCRs was positively correlated with intensity of PTSD symptoms. Finally, the circadian rhythm of cortisol was found to be normal in PTSD, with a trend toward loss of ultradian rhythms (small variations within the circadian rhythm): a finding suggesting a “hyperregulated” system.

Provocation tests

Dexamethasone is a synthetic glucocorticoid that deactivates the HPA axis through a negative feedback effect resembling that of cortisol. The Dexamethasone Suppression Test (DST) evaluates the magnitudes of the negative feedback loop that regulates the HPA axis by testing plasma cortisol levels following the administration of dexamethasone. The DST is used in endocrinology to evaluate the integrity of the HPA axis. Reduced cortisol suppression characterizes Major Depression, and the frequent association of depression and PTSD prompted a series of studies of the DST. Early studies revealed normal cortisol suppression in PTSD, possibly through

Table 22.3 Studies of the HPA Axis in PTSD

<i>Authors</i>	<i>Outcome measure</i>	<i>Population</i>	<i>Result</i>
<i>Peripheral glucocorticoids</i>			
Mason <i>et al.</i> , 1986, 1988	Urinary free-cortisol and NE Plasma cortisol	PTSD Vs. Major depression, Bipolar Affective Disorder, Paranoid schizophrenia	Lower cortisol level in PTSD. NE/cortisol ratio distinguishes PTSD from controls
Pitman and Orr, 1990	24-hr urinary-free cortisol and catecholamine	13 VnVs w/ PTSD and 10 healthy combat controls	Normal cortisol in PTSD
Yehuda <i>et al.</i> , 1990	Urinary cortisol	16 vietnam male combat veterans w/ PTSD and 16 normal controls	Lower mean cortisol level in PTSD
Resnick and Yehuda, 1994	Plasma cortisol	Rape victims in the ER	Lower cortisol level in victims of repeated rapes
<i>Glucocorticoid receptors</i>			
Yehuda <i>et al.</i> , 1991	Lymphocyte CGRS, plasma and 24 hours urinary cortisol	PTSD versus major depression, bipolar manic, survivors w/o PTSD	Larger number of GCRs in PTSD
<i>Provocation tests</i>			
Smith <i>et al.</i> , 1989	ACTH and cortisol response to CRF	8 VnVs w/ PTSD 11 normal controls	Lower ACTH response to CRF in PTSD
Yehuda <i>et al.</i> , 1993	Low dose DST	21 male VnVs w/ PTSD, 12 normal controls	Greater cortisol suppression in PTSD
Yehuda <i>et al.</i> , 1995	Plasma cortisol and lymphocyte GCR response to Dexamethason	14 VnVs w/ PTSD; 12 VnVs without PTSD, 14 Healthy controls	Larger number of GCRs and more suppression of cortisol by DEX in PTSD

Abbreviations: NE = Norepinephrine; EPI = Epinephrine; HR = Heart rate; BP = Blood pressure; AO = Monoamine oxidase; VnVs = Vietnam Veterans; PTSD = Post traumatic stress disorder; GCRs = Glucocorticoid receptors; DST = Dexamethason suppression test; ER = Emergency Room.

a floor effect. Using a smaller dose of dexamethasone, Yehuda *et al.* (1993) showed increased suppression of cortisol by dexamethasone in chronic PTSD. The extent of cortisol suppression was not related to the presence of comorbid depression.

Resnick and Yehuda (1995) have recently shown that shortly after rape, subjects who had previously been raped had lower cortisol levels, while subjects who were raped for the first time reacted by mounting a normal cortisol response. Cortisol levels in subjects with prior rape experiences did not correlate with the severity of the assault, while those of subjects without prior rape experience correlated significantly with the severity of the current assault. Given that repeated rape experience is associated with increased incidence of PTSD, it may be argued that survivors who tend to develop PTSD are those whose cortisol response to the trauma is abnormally low and who, because of such abnormal response, fail to properly terminate the acute response to stress, thereby increasing the effect of the stressor on the CNS (e.g., learning, memory consolidation).

PSYCHOPHYSIOLOGY

Responses to Cues Reminding of the Trauma

A first line of psychophysiological studies measured the physiological response (HR, skin conductance, frontalis muscles EMG, and plasma epinephrine) to cues reminiscent of the trauma in PTSD. Consistent findings of increased responsiveness to external (e.g., combat sounds) and internal (mental imagery) cues, and lack of such responses in trauma survivors without PTSD, provided a measurable evidence of phasic arousal in PTSD. These studies have also provided a "provocation test", which was used in later functional brain imaging studies of PTSD.

Auditory Startle Response

In animals and humans, sudden and intense auditory stimulation elicits a series of physiological responses known as an *acoustic startle response* (ASR). Firstly, a flexor motor response, properly called the *startle reflex*, occurs within 40–200 ms of stimulus onset. This response is typically measured in humans by recording the eyeblink. Autonomic responses (cardiac and electrodermal) follow, having longer latency (> 200 ms) and longer duration. Both components of the ASR habituate after repeated presentations of the same stimulus. Numerous neuronal afferents, including those from the *LC* and the *amygdala*, modulate the startle reflex and its habituation. Startle amplitude increases in the presence of a cue that had previously been paired with a shock. Conditioning of this type is often referred to as *fear potentiated startle* and is mediated by the central nucleus of the amygdala. Contextual cues can also modulate startle, and this modulation involves the bed nucleus of the stria terminalis and is further modulated by CRF.

Exaggerated startle, a DSM IV diagnostic criteria for PTSD, is reported by up to 86% of trauma survivors with the disorder. Reports of exaggerated startle have high specificity for PTSD in recent trauma survivors. In laboratory studies, however,

exaggerated eyeblink response was not consistently found in PTSD (Reviewed in Shalev and Rogel-Fuchs, 1993). Studies evaluating the autonomic component of the ASR, however, have shown an elevated cardiac and electrodermal response to auditory startle and slower habituation than of the electrodermal responses.

Autonomic habituation to startle is inherited and has been associated in humans with increased propensity to acquire conditioned responses. Lack of startle habituation, therefore, has been suggested as a genetic marker of PTSD. However, a recent prospective study has shown that subjects who develop PTSD do not differ from trauma survivors without PTSD in their startle responses one week after trauma. Differences between survivors with PTSD and other survivors develop over time such that four months after the trauma PTSD patients start to show the typical responses of chronic PTSD (Shalev *et al.*, in press). The role of startle habituation as a genetic marker was not supported by this study. Yet, the critical period during which physiological abnormalities that later characterize PTSD develop may be traced to the first four months following trauma.

MEMORY AND COGNITIVE FUNCTIONS

Several PTSD symptoms involve remembrance and learning, including: distressing recollections of the trauma; being easily reminded of the trauma; and behaving as if the trauma is recurring. For example: a traumatized war veteran can find himself constantly prepared to duck for cover while walking in a public park in a peaceful city. Another PTSD survivor would react to the sound of an ambulance siren by being suddenly flooded with images, smells, and body sensations related to a terrorist attack. Indeed, most PTSD subjects would report that they can remember the traumatic event in minute details years later, and that their recollections of the trauma have the quality of the "here and now" rather than that of a retrospective remembering.

Along with such forms of enhanced recollections, PTSD patients also complain of poor concentration, forgetfulness, difficulty following complex daily tasks, and difficulty learning new information. PTSD patients may also have difficulties to recall important fragments of their traumatic experiences. Dissociative episodes, during which the trauma is relived, are often seen in PTSD. Hence, both memory enhancement and impairment coexist in PTSD.

Responses to cues reminding the trauma in PTSD have been assimilated with conditioned fear responses in that they are involuntary, involve strong physiological activation, and are associated with intense negative affect and avoidance. Animal studies (Ledoux, 1995) suggest that the acquisition of conditioned fear responses is mediated by subcortical pathways involving the thalamus, the central and the lateral nuclei of the amygdala, and, by a parallel cortical pathway, the sensory cortex and the amygdala. Once acquired, memory traces of fear conditioning may not extinguish at subcortical levels, but rather be subjected to inhibitory control by cortical centers. Consider an example of classical conditioning in which a red light (conditioned stimulus, CS) is paired with footshock (unconditioned stimulus, UCS) to produce a conditioned response (CR). The above theory suggests that if the CR

decreases upon several unpaired presentations (extinction), it is because the relevant sensory and association cortex is blocking the response, not because the original association has been lost. Indeed, conditioned responses may be easily reinstated upon few presentations of paired CS and UCS. PTSD may represent a default of such cortical inhibition.

In PTSD patients CRs are particularly difficult to treat because of the frequent generalization of apprehension and avoidance to cues that are no longer directly associated with the trauma. For example, a rape victim may generalize her fear and avoidance to any man, weapon, or shadowed ally, rather than specifically avoid those that had direct bearing on her trauma. Such nonspecific response to remote reminders of the trauma has been associated with an imbalance between amygdala-mediated memory (i.e., emotional, generalized, and without spatiotemporal attribution), and hippocampus-mediated memory (more specifically linked to context and time).

Beyond the particularities of remembrance in PTSD, studies have shown that PTSD patients' attention is biased towards enhancing perception and remembrance of trauma-related cues (Zeitlin and McNally, 1991; McNally *et al.*, 1996). Studies have also suggested that an impairment of short-term verbal memory may exist in PTSD (Sutker *et al.*, 1991; Bremner *et al.*, 1992), but these findings are probably confounded by problems of attention due to anxiety and need further confirmation.

BRAIN IMAGING

Several animal studies suggest that chronic stress, through excess in brain corticosteroids, may lead to alterations in a category of hippocampal neurons, followed by cell death and a reduction in hippocampal volume. These studies have involved direct exposure to cortisol, as well as autopsy of monkeys living under stressful conditions (Sapolsky *et al.*, 1988, 1990). Hippocampal damage was also shown in humans who suffer from hypercortisolemia (Cushing's disease). Hypothetically, therefore, traumatic stress in humans may also lead to cortisol-mediated damage to hippocampal cells. The presence of memory dysfunction in PTSD, as described above, has been interpreted as further suggesting a dysfunction in hippocampus-related memory functions. Yet, other forms of extreme stress, particularly the repeated traumatization that often characterizes child abuse, are equal candidates for causing hippocampal damage.

Given these hypotheses, four brain imaging studies have assessed hippocampal volume in PTSD subjects and other trauma survivors. Bremner *et al.* (1995) compared hippocampal volume of 26 Vietnam veterans with PTSD with 22 healthy controls and found a reduced right hippocampal volume in PTSD. The volume of the right hippocampus in PTSD patients correlated significantly with the degree of verbal memory impairment as measured by the Wechsler Memory Scale. No correlation was found between verbal memory scores and volumes of other brain regions, nor between hippocampal volume and visual memory scores. Stein *et al.* (1995) found a 13% decrease in left hippocampal volume in survivors of child abuse; however, no correlation between hippocampal volume and verbal memory deficit

was observed. Finally Guzvitz *et al.* (1996) compared 7 Vietnam with PTSD with 7 healthy combat veterans and 7 matched healthy civilians. They found that the volume of both left and right hippocampi were significantly reduced in the PTSD group compared with the two non-PTSD groups.

Functional brain imaging studies have been performed using cues reminiscent of the trauma to elicit intrusive PTSD symptoms. During exposure to mental imagery of a trauma, an increase in cerebral blood flow was observed in the right limbic and paralimbic areas, the amygdala, the insula, and the anterior temporal lobe. In addition, area 18 was activated during mental imagery of the trauma and Broca's area was deactivated, the latter action suggesting an impairment in semantic structuring of memory in PTSD. The activation of the paralimbic area is common to other anxiety disorders.

DISCUSSION: STRESS THEORY AND PTSD

Historically, the field of traumatic stress evolved independently from the preexisting domains of stress and coping. Despite attempts to theoretically articulate "stress" and "traumatic stress" research, there has been very little interaction between the two fields. Indeed, the conjunction between the two is rather problematic. Early stress researchers have shown that excessive demands on the organism produce a typical sequence of physiological responses designed to keep the effect of the stressor on the organism within viable homeostatic boundaries. By analogy, the *psychological* responses to stress were also conceived of as regulatory mechanisms aimed to keep the *mental responses* within manageable boundaries. Traumatic stress, in contrast, is often conceived as a breach of such regulatory mechanisms, leading to "collapse of structures" and defenses. This claim, which clearly suggests a discontinuity between "normal" and "catastrophic" stress, must still be substantiated by research.

Stress research and the traumatic stress literature differ in a number of methodological dimensions. The stress literature is mostly experimental, using exploratory designs and controlled conditions. The traumatic stress literature, in contrast, is mostly naturalistic, retrospective, and observational. Traumatic stress researchers tend to use categorical outcome measures (mainly the development of a disorder), while stress research has been using mostly continuous outcome measures. Finally, most of the research on traumatic stress has been focused on evaluating the relationship between a trauma and subsequent disorders, thereby evaluating the "traumatogenic" nature of events rather than their "stressfulness."

Indeed, the stressors that trigger PTSD are ill defined at this point: PTSD can follow a single exposure (e.g., rape, car accident, or bush fire) as well as prolonged and dehumanizing atrocities, such as concentration camp experiences, captivity, or torture. The appropriateness of the acute, homeostatic stress model for PTSD has, therefore, been challenged, and the role of the triggering incident in the overall causation of PTSD is believed to be only partial. Indeed, the neurobiology of PTSD resembles that of chronic stress and includes alterations in both noradrenergic and HPA responses to challenge, suggesting a prolonged sensitization.

Proper understanding of the interface between stress and PTSD is crucial. PTSD is now at the forefront of mental disorders having consistent and replicable biological findings. PTSD, which is clearly triggered by a distinct event, constitutes a window of opportunity for exploring the effect of the environment on the CNS. PTSD, therefore, is likely to become the object of intense neurobiological research, testing many of the above hypotheses and extending the current findings.

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23 Effects of Glucocorticoids on Cognitive Performance in Humans: Evidence from Clinical Investigations and Experimental Studies

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Glucocorticoids (GCs) are an integral part of the body's endocrine stress response. The main GC produced by the human adrenal cortex is cortisol, in contrast to corticosterone in the rat. Like most other steroid hormones, cortisol can easily traverse the blood brain barrier and therefore exert direct effects on the central nervous system (CNS). This chapter briefly reviews literature concerning the effects of elevated GCs on cognitive performance in humans, with an emphasis on memory. In the first part of this chapter, clinical investigations that show associations between elevated GC levels and cognitive impairments in several diseases are summarized. In addition, the effects of chronic hypercortisolemia are documented. In the second part of the chapter, experimental studies that test the short-term effects of GCs on memory performance in healthy humans are presented. Finally, a short outline of possible neuronal mechanisms underlying the summarized findings is given.

The hippocampus, a component of the limbic system, contains the highest number of GC receptors in the brain. Animal studies have demonstrated that the hippocampus is an important regulatory site for the feedback action of the hypothalamus pituitary adrenal axis (HPA) (Jacobson and Sapolsky, 1991). There are two GC receptor types (type I or MR and type II or GR) which bind cortisol with different affinities. Whereas most of the type I receptors are occupied during the normal diurnal cycle, only elevated cortisol levels, as observed during stress, result in increased occupation of the type II receptors.

The hippocampus is also essential for certain memory processes. Animal studies and clinical investigations both show that the hippocampus is important for the so-called declarative or explicit memory, which includes semantic as well as episodic memory. Procedural or implicit memory, in contrast, which covers the domains of classical conditioning, priming, skill learning and nonassociative learning, is independent of the hippocampal formation (for review see Eichenbaum *et al.*, 1992; Squire, 1992). In addition, short-term memory is also hippocampal independent. The dichotomy between hippocampal-dependent declarative memory vs. hippocampal-independent procedural memory will be used to order and analyze the presented studies.

ELEVATED CORTISOL LEVELS IN CLINICAL DISEASES

Morbus Cushing

Cushing's disease is an endocrine disorder characterized by enhanced cortisol levels. This may be due to either excess pituitary ACTH production or enhanced adrenal cortisol secretion. Starkman *et al.* (1981) investigated 35 Cushing patients with a semistructured interview. In addition to other symptoms such as increased fatigue and depressed mood, 66% of their patients complained about concentration deficits and 83% reported memory problems. The same research group investigated Cushing patients with a neuropsychological test battery (Whelan *et al.*, 1980). Again they found that two thirds of the patients had neuropsychological impairments. The deficits were generally more frequent and severe in non-verbal, visual-ideational and spatial constructional abilities than in language and verbal reasoning. Martignoni and coworkers (1992) conducted a study with 24 Cushing patients and again found an impairment in verbal as well as nonverbal episodic memory in comparison to a control group, replicating findings from Starkman and coworkers. Interestingly Starkman *et al.* (1986) reported an improvement of psychiatric symptoms (e.g., depression and deficits in memory and concentration) in 23 patients with ACTH dependent Cushing's syndrome after pharmaceutical reduction of cortisol levels. These changes were also observed when ACTH levels remained high, indicating that cortisol, not ACTH, was the cause of the psychiatric impairment.

Depression

In affective disorders, especially in depression, there is often a dysregulation of the HPA axis. These patients have elevated basal cortisol levels and also show reduced negative feedback sensitivity of the HPA. For example they often do not suppress their endogenous cortisol secretion after application of the synthetic GC dexamethasone (DEX). These patients are therefore called DEX nonsuppressors. Several studies tried to link the endocrine status of depressed patients to their cognitive impairments. Rubinow and coworkers (1984) tested depressed patients and healthy controls with the Halstead Reitan Category test (a concept formation test which measures logical reasoning and not merely memory). They found a significant positive correlation between mean urinary free cortisol excretion and the number of errors in the category test in the depressed, but not control, patients. An even more robust correlation was observed, however, between age and error scores suggesting an age-cortisol interaction which might have been responsible for the cognitive deficits.

Another study (Wolkowitz *et al.*, 1990) demonstrated that depressed patients who did not show cortisol suppression to 1 mg DEX committed significantly more commission errors in a word-learning paradigm (declarative memory) compared to suppressors. Interestingly there was no difference between the depressed DEX suppressors and a healthy control group, suggesting that only the depressed nonsuppressors show cognitive impairments. This is in agreement with findings from

Winokur *et al.* (1987) who also observed memory deficits, delusion, and melancholic symptoms in depressive DEX nonsuppressors.

Dementia of the Alzheimer Type

Alzheimer's disease is also characterized by high cortisol levels and nonsuppression to DEX. De Leon *et al.* (1988) found a correlation between an enhanced cortisol response to the glucose tolerance test and cognitive decline. Heuser *et al.* (1988) reported a negative relationship between mean 24 hour cortisol levels and scores on neuropsychological tests. Oxenkrug and coworkers (1989) however found a similar relationship between post-DEX levels and scores in the Global Deterioration Scale in the female group only.

Cortisol Levels in Human Aging

Sonia Lupien and coworkers (1994) investigated the development of basal cortisol levels in 19 healthy elderly subjects over a three- to six-year period. They found that the slope of the change in cortisol levels over time predicted cognitive deficits. Correlational analysis showed a significant negative correlation between the slope and test performance for declarative memory (pair association) and selective attention. Those subjects who showed an increase in cortisol levels over the investigation period showed more pronounced cognitive impairment. Neither the last 24-hour cortisol level nor the averaged 24-hour level across the three measurements could predict the deficits.

O'Brien *et al.* (1994) found that post-DEX levels in healthy elderly subjects were inversely correlated with the results in a broad cognitive examination which covered orientation, language, memory, and praxis (Cambridge cognitive examination, CAMCOG). Subjects who failed to significantly suppress cortisol after DEX administration showed cognitive impairment. As in the study by Rubinow *et al.* (1984) with depressed patients, however, cortisol levels as well as the CAMCOG scores were confounded with age (i.e., older subjects had higher post-DEX cortisol levels and performed poorer in the CAMCOG).

Summary

The described clinical findings indicate that in a variety of disorders enhanced cortisol levels are associated with impaired cognitive performance in humans. The reported deficits appear to be broad and are often confounded with mood problems. However, these findings are only correlational in nature and therefore do not allow definitive statements about the cause of cognitive deficits in the patients. Do steroids really impair cognition or is the observed cognitive impairment only another symptom of a multifaceted syndrome unrelated to steroid levels? Furthermore, the studies reviewed above investigated the effects of chronically elevated cortisol levels on cognition. In order to test possible acute effects of GCs on cognitive performance in humans, we must turn to experimental studies in healthy subjects.

EXPERIMENTAL STUDIES

EEG Studies

One approach to study the effects of GCs on cognitive performance is the investigation of evoked electrical potentials to certain stimuli recorded in an electroencephalogram. The first study using this methodology in the context of CNS effects of GCs was performed by Kopell and coworkers (1970). Following infusion of a high dose of cortisol, they found a reduced amplitude of visually evoked EEG responses to light flashes in the EEG components P170 and N200. Similar results were obtained by Born *et al.* (1987). In a dichotic listening paradigm, cortisol reduced the N1 component of the EEG and also inhibited the mismatch negativity (the difference amplitude between the event-related potentials to standard and deviant target pips). However, in another study the same research group found an increase in the vertex potential component of the auditory evoked potential after cortisol application (Born *et al.*, 1988). The authors interpreted their findings as evidence for different cortisol action at different sites of the brain (reticular formation vs. cortical structures) and at different levels of the information process (early vs. late stages).

Neuropsychological and Memory Studies

A second approach to investigate the effects of corticosteroids on cognitive functions in humans is to acutely administer these steroids to healthy individuals and then to test for alterations in their cognitive performance. In the past both synthetic GCs (e.g., DEX, prednisone) as well as endogenous GCs (e.g., cortisol, corticosterone) were used to assess the impact of these steroid hormones on cognitive functions.

Studies using synthetic GCs

Wolkowitz *et al.* (1990) performed two studies in which they investigated the effects of the synthetic steroids DEX and prednisone on the performance in a word-learning paradigm (declarative memory). The subjects were presented with a list of 12 words and after a 90-sec distraction task, free recall as well as correct recognition was tested. In the first study the effect of a premedication with DEX on memory performance was evaluated in a group comparison design. After ingesting 1 mg of DEX the night before testing, subjects from the treatment group made significantly more commission errors (falsely recalled words or intrusions) with no significant change in the number of omission errors. There were no differences between the placebo group and the treatment group in measures of attention, correct free recall, and total correct recognition.

In a second study the effect of prednisone (80 mg over five days) was tested. The memory test was similar to the one described in the first study. In this experiment a repeated measurement design was used with three test sessions (baseline, after prednisone, after washout). The authors again found a significant increase of commission; however, errors were present in the recognition task, not in the free recall condition. Subjects under prednisone more often identified distractors as target words.

No differences were found in the other measures. Wolkowitz and coworkers interpreted their findings as evidence for a specific GC-induced impairment to discriminate previously presented relevant information from irrelevant new information.

Newcomer *et al.* (1994) tested the effects of DEX on cognitive functions in healthy subjects. They received DEX or placebo for 4 days. There were four test sessions (baseline, after one day of treatment, after four days of treatment, and one week after the end of treatment). The test session included a paragraph-recall test for the assessment of verbal declarative memory, a serial-addition task for the assessment of attention, a vigilance test, and a test for visuoperceptual functions. The authors found a significant impairment in the declarative memory task after four days of DEX, but not after one day. Subjects receiving DEX for four days recalled less information from the presented paragraph. No increase in commission errors was observed, in contrast to Wolkowitz *et al.* (1990). Newcomer and coworkers argue that their findings could present evidence for a GC impairment of hippocampus-mediated memory processes, whereas more basic cognitive functions (e.g., attention, vigilance) seem to be spared. They speculate that a single overnight application of DEX might not be sufficient to significantly increase DEX levels in the brain and that a longer treatment period would be necessary to produce DEX effects on cognitive functions. Their hypothesis is supported by animal receptor-binding studies, which show that after a single administration of DEX only small amounts of DEX bind to glucocorticoid receptors in hippocampal tissue (Miller *et al.*, 1992).

In discussing the results of studies that investigate possible effects of DEX on cognitive functions, one should note that DEX is a potent negative feedback signal for cortisol production. Therefore, it is almost impossible to ascribe observed changes in cognitive performance to either direct effects of DEX on CNS structures or to secondary effects following the significant reduction of circulating cortisol levels.

Studies using endogenous GCs

Beckwith *et al.* (1984) tested the effects of different doses of cortisol (0, 5, 10, 20, or 40 mg) on an immediate recall test. Subjects were presented with a tape recorded list of 12 words. Each subject was presented with eight different lists at two different speeds of presentation. Each successive two lists were defined as one level of practice. Serial effects were also investigated. The primacy effect represents long-term memory portions of recall, whereas the recency effect reflects short-term memory content. The authors found at the first level of practice that the 5-, 10-, and 40-mg doses showed a facilitating effect on memory performance in comparison to placebo; no such effect was observed at the 20-mg dose. No effects were obvious at the second level of practice. At the third level of practice 40 mg improved whereas on the fourth practice level 5 mg impaired performance. Beckwith *et al.* interpreted their findings as evidence for a dose-dependent effect of cortisol. Since they did not find any interaction between cortisol and serial position effects (primacy vs. recency), the authors argue that their results are more consistent with a cortisol-induced increase in arousal or motivation than with an enhancement of more specific memory functions (long-term vs. short-term memory).

The use of different GC doses in the study of steroid effects on memory performance is a valuable approach. However, an immediate recall task cannot test memory types with respect to hippocampal involvement. The interpretation of the Beckwith study is obfuscated by the fact that cortisol was administered together with glucose, a substance which has inherent, potent memory effects. Moreover, the amount of recalled words was generally low, possibly indicating poor subject motivation. More definitive negative findings were reported in a similar study (Fehm-Wolfsdorf *et al.*, 1993). In a crossover design they tested the effects of 50 mg cortisol vs. placebo bid (morning and evening). Subjects under cortisol remembered the same number of words as did subjects under placebo. There was a trend towards better performance in the morning under both conditions.

In a more elaborate study Lupien *et al.* (1995) infused young subjects with cortisol over a period of 100 minutes. In addition to placebo they used three different doses of cortisol (40 µg/kg/h; 300 µg/kg/h; 600 µg/kg/h). Subjects were tested for declarative memory performance in a word-pair learning paradigm with cued recall test. Subjects were tested first during the infusion period and then four hours later using a different word list. Four days after the treatment, subjects were again tested for delayed recall of the two lists (one learned during infusion and one learned four hours later). When the four groups were compared by ANOVA no difference between groups was found. However a significant negative correlation emerged between the cortisol increase following cortisol infusion and the amount of words recalled four days later. Subjects who showed the greatest increase in cortisol during infusion recalled less words from the list four days later. Lupien and coworkers suggested that their findings could indicate that the effects of corticosteroids on memory performance may be delayed in time and may especially weaken acquisition.

Our research group performed two studies in order to investigate the effects of cortisol on memory performance (Kirschbaum *et al.*, 1996). In the first study we intended to test for a relationship between the amount of cortisol secreted in response to psychosocial stress and performance in a declarative memory task after stress cessation. We hypothesized that there would be a negative correlation between acute stress-induced cortisol levels and memory performance. Thirteen healthy adults were first exposed to a standard laboratory stressor, the 'Trier Social Stress Test' (TSST), which mainly consists of a five-minute public speaking task with an additional five minutes of mental arithmetic before an audience and a video camera (Kirschbaum *et al.*, 1993). Before and after the stressor, saliva samples were obtained from the participants in order to determine free cortisol levels. Ten minutes after the end of the TSST, subjects had to learn a word list consisting of 24 nouns. After a distraction task, subjects were tested for cued recall performance. As expected the TSST led to a significant increase in cortisol levels. Most importantly, however, there was a highly negative correlation ($r = -0.70$) between the cortisol increase in response to the stressor and the number of correctly recalled words.

Since a large number of psychological and endocrine factors change in response to the TSST, the results of the first experiment cannot prove that a change in cortisol levels impairs performance in a declarative memory task. Therefore, a second experiment was conducted. Forty healthy men received placebo or 10 mg

cortisol one hour prior to memory testing. Memory tests consisted of a word-learning task with a priming test for the assessment of procedural memory, and a cued recall test for the assessment of declarative memory performance. Between the learning and recall phases, subjects also had to work on two spatial memory (mental rotation) tasks. In the first spatial memory task ("park task") subjects had to read a description of a walk through a park with the task to describe the way back. In the second task ("barn task") subjects had to read a description of a scene in a barn and 10 objects were described in relation to the subject's spatial position. After learning this description subjects had to imagine a 90-degree rotation of themselves in the barn and then provide the location of the 10 objects relative to their "new" location. These two tasks were employed to assess more complex forms of declarative memory. Moreover, they were included since results from animal studies suggest that the hippocampus is quintessential for spatial orientation. Thus, it was expected that the cortisol treatment would interfere with the subjects' spatial memory.

Ingestion of 10 mg cortisol led to a sixfold increase in free cortisol with mean values at the upper physiological level (cortisol group: 64.2 ± 9.8 SEM; placebo group: 9.3 ± 0.7 nmol/L). The cortisol group recalled significantly fewer words from the word list under the cued recall condition with no difference between the two groups in the priming task (procedural memory). In the two spatial memory tasks the cortisol group again showed a poorer performance reflected in more errors in the 'park' and in the 'barn' tasks. Taken together our findings demonstrate that a single administration of cortisol (10 mg) can impair both simple and complex forms of declarative memory performance, whereas procedural memory performance as assessed with a word-stem priming task seems to be spared. Stress-induced increases in cortisol levels may therefore be a viable candidate for reduced declarative memory function following exposure to psychologically distressing events or tasks.

POSSIBLE NEURONAL MECHANISMS UNDERLYING THE OBSERVED FINDINGS

The question how GCs might interfere with memory performance will only be addressed briefly here since a detailed description of the possible mechanisms involved would be beyond the scope of the present chapter.

One possible neuronal mechanism underlying memory formation is the so-called long-term potentiation (LTP) of hippocampal pyramidal cells. Stress in animals was shown to decrease LTP and there is an inverted U-shaped function between GC levels and LTP (Diamond *et al.*, 1992), indicating that extremely low or high GC levels can reduce neuronal plasticity. Evidence has accumulated that while an occupation of the type I receptor promotes LTP induction, enhanced occupation of the type II receptor (e.g., following stress or pharmacological treatment with GCs) seems to impair LTP (for review see McEwen and Sapolsky, 1995). In addition to receptor-mediated genomic effects of GCs, nongenomic membrane effects of cortisol or one of its metabolites should be taken into account in discussing GCs effects on CNS functions (McEwen, 1991).

SUMMARY AND OUTLOOK

In this chapter we summarized some evidence to suggest that high circulating levels of cortisol in clinical diseases or due to psychosocial stress can be associated with cognitive impairment. While clinical studies provide a correlational link between GCs and cognitive functions, experimental studies were able to demonstrate that short-term treatment with different GCs can cause impaired cognitive functioning. Hippocampus-mediated declarative memory seems to be particularly influenced by these steroids. In contrast, performance on a nondeclarative (hippocampus-independent) memory task does not appear to be influenced by GCs. Future studies should address the question whether the memory formation process and the ability to recall stored information are differentially affected by GCs.

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24 Blood–Brain Barrier in Stress: A Gateway to Various Brain Diseases

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The breakdown of the blood–brain barrier (BBB) has long been recognised as one of the important feature occurring in wide variety of neurological and psychiatric brain diseases. However, it is not clear whether the breakdown of the BBB that occurs in many different brain diseases is beneficial or harmful in nature with regard to the brain function and cell reaction. This review is based on experimental evidences in heat stress which suggest that opening of the BBB is closely related with neuronal damage and pharmacological blockade of barrier breakdown following heat exposure is neuroprotective. These observations indicate that the BBB can be regarded as a gateway of various neurological diseases and brain pathology.

THE CONCEPT OF A BLOOD–BRAIN BARRIER

The BBB is a physiological dynamic barrier which resides mainly in the endothelial cells of cerebral capillaries (Rapoport, 1976). The endothelial cells of cerebral capillaries are connected to each other via tight junctions and possess a high electrical resistance of about $2000\text{ ohm}^{-1}\text{ cm}^2$ (Olesen and Crone, 1986). The endothelial cells of cerebral capillaries do not contain vesicles for transendothelial transport and are surrounded by basal lamina and glial cells (Bradbury, 1990). These characteristics make the cerebral capillaries almost impermeable to most substances constituting the permeability properties of the BBB very similar to that of an extended plasma membrane. However, the transport of nutrients, ions and hormones which are essentials for brain functions are regulated between blood and

brain via specific transport system comprising molecule transporters and receptors, ionic pumps, and various enzymes present into the endothelial cells (Greenwood *et al.*, 1995). Recent research revealed that the BBB function can also be influenced by metabolic changes in the brain, altered neuronal activity as well as changes in the periphery such as circulating levels of hormones, neurochemicals and cytokines (Bradbury, 1990; Greenwood *et al.*, 1995) indicating that the maintenance of the BBB *in vivo* is a complex phenomena.

Blood-Brain Barrier in Normal and Pathological Conditions

The BBB remains tight all the time in normal physiological conditions because tracers ranging from 5 Å to about 85 Å (Stoke's radius) comprising about 500 kD to 150,000 kD (molecular weight, MW) do not pass the normal BBB. However, the integrity of the BBB is compromised under wide variety of experimental and in pathological conditions (Rapoport, 1976; Wahl *et al.*, 1988; Bradbury, 1990; Johansson *et al.*, 1990) (Table 24.1).

Table 24.1 Brief summary of various experimental and disease conditions in which the BBB is disrupted to various tracers

<i>Disease conditions</i>	<i>BBB tracers</i>	<i>Possible mechanisms of tracer transport</i>
<i>Neurodegeneration</i> Alzheimer's disease, brain tumors schizophrenia, dementia, ischemia, infarction peripheral nerve lesion	Serum proteins HRP microperoxidase Lanthanum radiotracers	vesicular transport endothelial cell permeability
<i>Trauma</i> mechanical ^a , hypoxia, hyperoxia, ischemia, metabolic insults, incision ^b	HRP, radiotracers Evans blue Trypan blue Lanthanum	vesicular transport widening of tight junctions ^a endothelial cell permeability
<i>Influence of chemicals</i> serotonin ^b , histamine protamine, norepinephrine, 5-HTP ^b , bradykinin, prostaglandins, leukotrienes, glutamate, L-NAME, hyperosmotic solutions ^a	HRP, Evans blue Lanthanum, microperoxidase radiotracers	vesicular transport widening of tight junctions ^a endothelial cell permeability
<i>Vascular diseases</i> hypertension ^a (mechanical, chemical or metabolic) hypotension	radiotracers, Evans blue, HRP, Lanthanum	widening of tight junctions ^a endothelial cell permeability vesicular transport
<i>Stressful situations</i> immobilization ^b , forced swimming ^b , heat exposure ^b , seizures, training in water maze, adrenalectomy	HRP, Evans blue radiotracers Lanthanum	endothelial cell permeability vesicular transport

Compiled from various sources; ^a known to occur; ^b authors own investigations.

It seems quite likely that the BBB plays an instrumental role in precipitating abnormal brain function probably via alterations in fluid microenvironment of the CNS.

Blood-Brain Barrier in Stressful Conditions

Investigations on the BBB in stressful situations has largely been ignored in the past. The concept of increased BBB permeability in stressful conditions is solely based on the investigations done by Sharma and Dey (1981) who reported for the first time that prolonged immobilization has the capacity to induce breakdown of the BBB to protein tracers in various brain regions. Subsequent experiments done by this group further revealed that stress of forced swimming and heat exposure is also associated with marked increase in the BBB to various tracers in specific brain regions (Sharma, 1982; Sharma and Dey, 1986; Sharma *et al.*, 1991a,b, 1992a,b, 1994, 1996a). These observations for the first time showed that various endogenous stressful conditions have the capacity to induce a breakdown of the BBB which seems to be very selective and specific with regard to the various brain regions under a particular type of stress. This gives an idea of selective vulnerability of the BBB in specific brain regions activated by stress, a feature which is now well recognised in other experimental conditions such as ischemia, trauma and infarction (Johansson *et al.*, 1990).

Biochemical studies from Sharma and his co-workers further showed that the increased permeability of the BBB in stress conditions is well correlated with the increased levels of plasma and brain serotonin (Sharma, 1982; Sharma and Dey, 1986, 1987), a neurotransmitter which is involved in wide variety of neurological and psychiatric diseases (Essman, 1978). These studies provided an important link between breakdown of the BBB occurring in a wide variety of neurological diseases with circulating vasoactive substances and opened a new vista of research regarding a neurochemical basis of the BBB permeability that could be mediated via various neurotransmitter receptors and second messenger system (Wahl *et al.*, 1988; Bradbury, 1990; Black, 1995; Greenwood *et al.*, 1995). Recent studies in many laboratories now support this as a valid concept in both *in vivo* and *in vitro* models (Olesen and Crone, 1986; Bradbury, 1990; Black, 1995; Greenwood *et al.*, 1995).

Chemical Mediators of the BBB

As a follow up of the observations of Sharma *et al.* in early eighties, several investigators examined the influence of different vasoactive substances on the BBB function by applying different neurochemicals on both the luminal and abluminal side of the cerebral microvessels (Wahl *et al.*, 1988; Bradbury, 1990; Black, 1995; Greenwood *et al.*, 1995). A survey over of chemical mediators of the BBB is shown in Table 24.2. Though the list of chemical mediators of the BBB is still expanding, it is quite interesting to note that most of these compounds are also involved in vasogenic edema formation (Wahl *et al.*, 1988). Edema is one of the common complication of many brain diseases leading to brain pathology that could be life threatening particularly following trauma, ischemia or infarction in which extravasation of plasma proteins is quite extensive following the primary insult (Rapoport, 1976).

Table 24.2 Chemical mediators of BBB dysfunction

<i>Chemical mediators</i>	<i>Receptors mediated</i>	<i>BBB permeability tracers</i>	<i>Signal transduction mechanisms</i>	<i>Vasogenic edema</i>
Bradykinin	B ₂ -kininergic	Na ⁺ -Fluorescein	PI, PKC	Yes
Arachidonic acid	—	Na ⁺ -Fluorescein FITC-dextran EBA	—	Yes
Free radicals	—	EBA, Na-fluorescein	—	Yes
Leukotrienes	—	EBA, Na-fluorescein	—	?
Cytokines	—	Na-fluorescein	—	?
Histamine	H ₂	EBA, Na-fluorescein FITC-dextran, HRP	cAMP	Yes
Serotonin ^a	5-HT ₂	EBA, HRP, La ⁺⁺	cAMP	Yes
Endothelin	ET ₁	⁵¹ Cr, ⁴⁵ Ca	Prostanoids cAMP, Ca ⁺⁺	?
Nitric oxide ^a	—	MEAP, EBA	—	Yes
Dynorphin ^a	—	EBA, La ⁺⁺	—	Yes

Compiled from various sources, ^a authors own investigations.

Crone and Olesen group in Denmark (Olesen and Crone, 1986) using isolated frog microvessels meticulously showed that the BBB is very tight under normal condition and has a very high electrical resistance (about 2000 ohm per cm²) which closely correlates with the tightness of the barrier. Application of various vasoactive substances in this model decreases the electrical resistance which is related with the increased BBB permeability and this phenomenon seems to be receptor mediated.

Routes of Leakage of the BBB

The possible routes of tracer transport across the cerebral endothelium in pathological conditions are still controversial. Electron microscopical studies of the cerebral endothelium under normal and pathological conditions showed that the permeability of the BBB is increased under a wide variety of conditions by an increase in the number of vesicular profiles. Observations in various experimental and clinical studies also favour this hypothesis that BBB breakdown occurs via transendothelial cell transport (Bradbury, 1990; Greenwood *et al.*, 1995). However opening of the tight junctions are also important under some conditions (Rapoport, 1976; Johansson *et al.*, 1990). Thus, hyperosmotic shrinkage of the cerebral endothelium or increase in transmural pressure in the cerebral microvessels by hypertension either produced by chemicals or by physical means disrupts the tight junctions (Johansson *et al.*, 1990). However, the permeability of endothelial cell membrane is also increased under these conditions apart from widening of the tight junctions (Bradbury, 1990). It may be that various neurochemicals influence the endothelial cell membrane or junctional permeability via a receptor mediated mechanisms, a feature which require additional investigations.

THE CONCEPT OF STRESS

Stress is defined as a non-specific response of the organism following altered situation (Selye, 1976). This definition is still valid for secretion of hormones, gastric ulcers and some non-specific response of the autonomic nervous system. However, the concept of stress as a non-specific response is changing with the rapidly advancing knowledge of the molecular mechanisms of the central nervous system (CNS). Recent evidences show that various neuronal population can selectively be activated by different kinds of stressors (Hughes and Dragunow, 1995). Thus, *c-fos*, a marker of neuronal activation can be upregulated in specific brain regions following immobilisation, running and other diverse kinds of various stressors (Hughes and Dragunow, 1995). Similarly, response of heat shock proteins (HSP), the so-called stress proteins, is also activated in selectively vulnerable neurons and sometimes in glial cells following trauma, ischemia and hyperthermia (Sharma *et al.*, 1992a,b, 1995). These molecular markers of stress reactions are very selective in specific regions of the CNS depending on the magnitude and duration of a particular stress stimuli (for details, see Hughes and Dragunow, 1995). This indicates that the concept of stress as a non-specific response of the body to the altered demand needs to be redefined in the light of selective molecular markers of the CNS.

Stress Influences Brain Function

The basic unit of the CNS is the neuron which can discriminate changes in the environment in response to different stimuli. Depending on the conditions, neurons can show short- or long-lasting changes in response of the altered environmental conditions due to existence of universal stimulus-response information processing mechanisms. The responses of neurons to the environment may be divided into early and late phases. The *early* response that last between milliseconds to minutes are brought about by changes in first messengers like neurotransmitters or growth factors. These substances then act on cell surface receptors by activation of second messenger system such as specific protein kinases which in turn actively phosphorylate specific neuronal proteins. The *late* response which occurs within hours and days involve gradual and permanent changes in the neurons. Changes in gene expression is necessary for this kind of response which is caused either directly using first messengers by hormone receptor complex interaction or indirectly via second messenger system involving interaction with cellular DNA in order to regulate and/or alter gene expression (Hughes and Dragunow, 1995).

Stress Induces Brain Diseases

There are now scientific evidences that sensitisation to stressors are encoded at the level of gene expression. This alteration in gene expression will in due course of time eventually manifest into major affective brain disorders (Hughes and Dragunow, 1995). Various kinds of brain diseases such as Alzheimer's diseases, schizophrenia, other kinds of neurodegenerative diseases and mental abnormalities are classified as

long-term stress disorders. This is evident with the fact that the upregulation of various kinds of stress proteins in these diseases has been demonstrated. Similarly, many other forms of traumatic, hypoxic or ischemic injuries are also associated with upregulation of stress proteins. These findings strongly demonstrate that stress occurring either at the cellular level or at the system level plays an important role in inducing various brain diseases.

Neurochemical Basis of Brain Diseases

Recent evidences strongly favour the idea that stress induced alterations in neurochemicals has the capacity to influence gene expression and thus induce short-term or long-term encoding of the stress experience which in turn can lead to reversible or permanent changes in the brain function (Hughes and Dragunow, 1995). Current data indicate that almost all neurochemicals and their metabolites are altered under stressful situation (Hughes and Dragunow, 1995). These altered chemical compounds may influence electrical, chemical, vasomotor and metabolic activities of the CNS at specific regions depending upon the availability of various receptor sites at the neuronal, glial or vascular level. After binding to the receptor sites these neurochemicals activate second messenger systems and alter the intracellular mechanisms via various signal transduction pathways (Hughes and Dragunow, 1995). The most widely studied neurochemicals in stress and brain diseases are biogenic amines, neuropeptides, excitatory as well as inhibitory amino acid neurotransmitters and their receptors (Hughes and Dragunow, 1995) however, the relationship between altered metabolism of various neurochemicals and neuronal dysfunction is still unclear.

Serotonin as a Mediator of Stress, BBB Permeability and Neurological Diseases

Serotonergic transmission in the CNS appears to be most vulnerable in various stress paradigms resulting in altered metabolism of serotonin in the periphery as well as in the CNS. Altered metabolism of serotonin occurs in the blood, brain and CSF under a wide variety of brain diseases (Essman, 1978). Recent evidences suggest that in fact serotonin can be regarded as a stress hormone (Hughes and Dragunow, 1995). This is further supported by recent findings from our laboratory that the amine is involved in stress protein response and *c-fos* gene expression in the CNS following stress induced by trauma or hyperthermia (Sharma *et al.*, unpublished observations). Thus, inhibition of serotonin synthesis with p-chlorophenylalanine (p-CPA) prior to spinal cord injury attenuated HSP-72 kD expression in the spinal cord and markedly reduced the upregulation of *c-fos* in the sensory and motor neurons following traumatic stress produced by a focal lesion of the rat spinal cord. Experimental evidences generated in the past from our laboratory show that the amine is a mediator of BBB permeability and has the capacity to induce brain edema formation and cell changes. Thus, infusion of small amounts of serotonin (10 µg/kg/min) into the blood stream for 10 min increases the BBB permeability, attenuates regional cerebral blood flow (CBF) and induces neuronal desynchronisation as evidenced by the

disturbances in the spontaneous electroencephalogram (EEG) activity (Sharma *et al.*, 1990; Winkler *et al.*, 1995). It appears that serotonin induced BBB disruption in many brain regions is an instrumental factor for the development of edema formation and cell changes. This hypothesis is supported by the fact that drugs which attenuate or prevent serotonin induced BBB disruption also reduced the edema formation and cell changes (Sharma *et al.*, 1996b).

Disturbances of the BBB in Brain Diseases

Our hypothesis that the BBB plays a detrimental role in brain pathology and brain diseases is well supported by many recent clinical and experimental observations. Thus, the BBB breakdown occurs in many kinds of brain diseases involving brain pathology (Rapoport, 1976; Bradbury, 1990; Greenwood *et al.*, 1995). Since the BBB is a physiologic dynamic barrier which maintains a constant composition of the cerebral microenvironment (Rapoport, 1976), alteration in this will result in disturbances of neuronal function. Breakdown of the BBB will result in the exposure of CNS to various immunologic, ionic, chemical and vasoactive components from which it is prevented before due to an intact barrier. As a consequence to this, a series of adverse reactions will occur that can lead to abnormal brain function (Sharma *et al.*, 1992a,b). During the last two decades we have focused our interest in stress induced abnormal brain function via an opening of the BBB in the rat. Experiments carried out in our laboratory revealed that increased BBB permeability following immobilisation stress is capable to induce EEG abnormalities at the time of BBB opening (Sharma and Dey, 1988). The desynchronised EEG changes can be compared to those found in a wide variety of mental illness and in serious neurological diseases. These observations point out that opening of the BBB in stress is harmful and may induce abnormal brain function which in due course will result in profound cell injury.

PROBLEMS OF HEAT STRESS

The problems of heat stress and heat stroke is known since Biblical times (Judith 8: 2-3) however the detailed mechanisms behind brain damage caused by heat exposure is still poorly understood. Heat injury is the most severe illness caused by high ambient temperature to the human population and is the third largest killer in the World after the cardiovascular and traumatic injuries to the CNS (Ellis, 1972; Sminia *et al.*, 1994).

Heat stroke may result following heat associated with high fever or marked exertion at high ambient temperature. This is mainly because of the inability to dissipate metabolically generated heat production. When the body temperature reaches beyond 41°C or higher, development of hypotension and metabolic brain dysfunction will lead to coma (Sminia *et al.*, 1994). A reduction in the effective circulating blood volume leading to cardiovascular dysfunction and respiratory distress will generate hypoxic brain injuries that can be the cause of death in more than 50% of the heat stress victims (Ellis, 1972; Sminia *et al.*, 1994). There are only a few post-mortem

studies on human brain from heat stress victims published 40–50 years ago using light microscopy. These reports suggest that the CNS is one of the most vulnerable region following hyperthermia (Malamud *et al.*, 1946). However, these authors did not examine different brain regions systematically, such as hippocampus and the ultrastructure of the CNS was not investigated. At that time the scientific knowledge and significance of the BBB function was not well identified. Thus, microvascular permeability and edema were largely ignored. Understanding of neuronal, glial and myelin pathology using new technology and specific molecular markers of the CNS at both light and electron microscopy in relations to the BBB dysfunction and edema formation following heat stress is thus highly needed.

Our Investigations in Hyperthermic Brain Injury

We initiated a series of investigations in our laboratory on the effects of heat induced damage of the CNS in a rat model using multi-disciplinary approach (Sharma, 1982; Sharma and Dey, 1987). In our model, rats were exposed to heat at 38°C in a biological oxygen demand (BOD) incubator and the relative humidity (45–51%) and wind velocity (20–26 cm/sec) were kept constant. This experimental condition is quite comparable to that of clinical situation (Sharma and Dey, 1987).

Stress Symptoms

Subjection of rats to heat induces stress symptoms. Thus rats exposed to 1 h, 2 h and 4 h heat stress showed a linear rise in their body temperature and the behavioural symptoms such as salivation (a measure of activation of heat loss mechanisms) and prostration (a sign of heat exhaustion) were maximum at the end of 4 h heat exposure (Sharma, 1982; Sharma and Dey, 1987). Gastric ulceration and micro-haemorrhages in the mucosal wall of the stomach were present in all the animals at the end of 4 h heat stress (Sharma and Dey, 1987). These observations point out that the model offer good possibilities to study the influence of heat on the structure and functions of the CNS.

BBB and Cerebral Blood Flow in Heat Stress

The intact BBB is an important indicator of normal brain function. Thus, we examined the BBB function to macromolecular protein tracers using Evans blue albumin and radioactive iodine [¹³¹I]–sodium in various brain regions following heat stress. These tracers when administered into the blood stream will bound to serum albumin. Leakage of the tracers across the cerebral endothelium thus represents extravasation of tracer-protein complex (MW 40–70,000 kD). The passage of tracer across the cerebral endothelium was examined at ultrastructural level using lanthanum hydrochloride. Lanthanum is an electron dense ionic tracer (MW 70 kD) which can easily be visualised as dark black particles under transmission electron microscope (Sharma and Cervós-Navarro, 1990). The results show that subjection of rats to a 4 h HS resulted in a marked increase in the BBB permeability to Evans

blue and [131 I]-sodium tracers in various brain regions. This increase in the permeability of the BBB was most marked in young rats. However, this increase in the BBB permeability was not evident in young animals subjected to 1 h or 2 h periods of heat stress. These observations indicate that duration of heat stress and age are important factors in heat induced breakdown of the BBB permeability. The regional BBB permeability showed marked variations in the degree of the BBB opening in heat stress (Sharma and Dey, 1987). This difference in the BBB opening could be due to difference in the various neurochemical receptor subpopulation on the microvessels, differences in the neuronal mechanisms responding to heat stress or differences in the local cerebral circulatory or metabolic demands, a feature which require additional investigation. Although the regional CBF showed a marked decline at the time of BBB opening, the magnitude of the regional CBF reduction was not correlated to the degree of regional increase in the BBB permeability (Sharma and Dey, 1987). This indicates that regional ischemia alone is not involved in the mechanisms of the BBB opening (Figure 24.1). It appears that stress associated with heat plays an important role in the BBB breakdown because urethane anaesthetised animals when exposed to similar heat stress did not exhibit either stress symptoms or breakdown of the BBB.

The passage of tracer across the cerebral endothelium in heat stressed animals seems to occur via an increase in the transendothelial transport rather than widening of the tight junctions (Sharma *et al.*, 1994). We did not observe any open tight junctions in our investigation on more than 250 vascular profiles randomly selected from different brain regions showing increased permeability of the BBB to lanthanum. On the other hand, infiltration of lanthanum can be seen within the endothelial cells. In some microvessels, the number of vesicular profiles loaded with lanthanum are found attached to both the luminal and abluminal side of the cerebral endothelium (Sharma *et al.*, 1991a). In almost all cases where the lanthanum was present in basal lamina, the endothelial cells can be seen completely filled with the tracer (Sharma and Cervós-Navarro, 1990).

Serotonin Metabolism in Heat Stress

We used a specific and highly sensitive fluorometric assay for the determination of serotonin in the plasma and brain of rats exposed to heat (Sharma and Dey, 1987). Our results showed a good correlation between increased levels of plasma and brain serotonin levels and increased BBB permeability in the stressed animals (Sharma, 1982; Sharma and Dey, 1987). Thus animals exposed to 4 h of heat stress showed a several-fold increase in the plasma and brain levels of the amine at the end of heat exposure. On the other hand, animals exposed to a short duration of heat or anaesthetised animals subjected to 4 h heat stress did not show abnormalities in the serotonin activity compared to control rats (Figure 24.1). Further evidence of a close relationship between serotonin and the BBB permeability came from the study on animals exposed to chronic heat exposure. Thus when animals exposed to 1 h heat daily for 7 days their plasma serotonin levels was found to be elevated on the 7th day. When these rats were subjected to additional 4 h heat stress on the 8th day, they

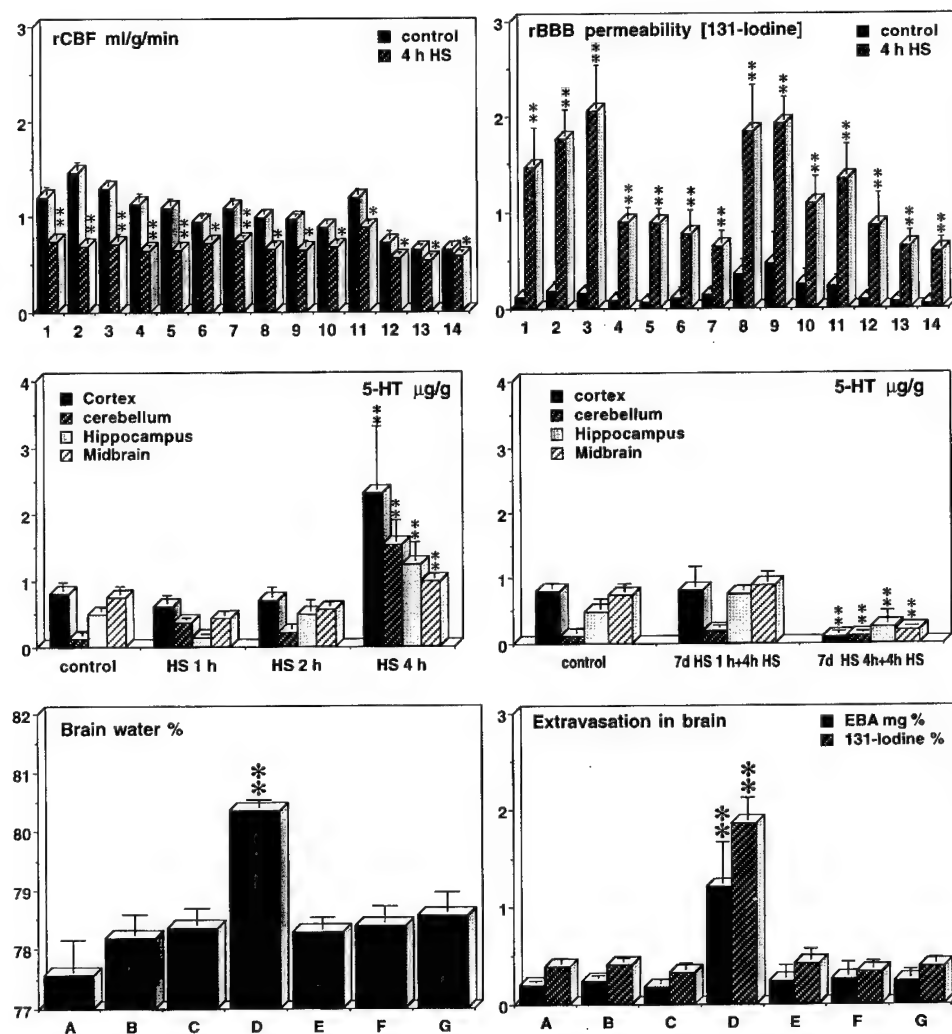


Figure 24.1 Regional cerebral blood flow (left), blood-brain barrier permeability (right) following acute heat exposure (upper panel); regional brain 5-HT concentration following acute (left) and chronic (right) heat exposure (middle panel); and brain water content (left), Evans blue and 131 I-sodium extravasation (right) in the whole brain following acute and chronic heat exposure in conscious young rats. * $P < 0.05$, ** $P < 0.01$, Student's unpaired t -test. 1. frontal cortex, 2. parietal cortex, 3. occipital cortex, 4. temporal cortex, 5. cingulate cortex, 6. hippocampus, 7. caudate nucleus, 8. thalamus, 9. hypothalamus, 10. sup. colliculus, 11. inf. colliculus, 12. cerebellum, 13. pons, 14. brain stem; A. control, B. 1 h, C. 2 h, D. 4 h acute HS in conscious rats, E. 4 h HS in urethane anaesthetised rats, F. chronic HS 1 h for 7 days + 4 h HS on the 8th day, G. chronic HS 4 h for 7 days + 4 h HS on the 8th day; EBA = Evans blue albumin, HS = heat stress, 5-HT = 5-hydroxytryptamine (serotonin), rBBB = regional blood-brain barrier, rCBF = regional cerebral blood flow.

did not show any further increase in the level of this amine (Figure 24.1). In these rats, occurrence of stress symptoms or increased BBB permeability was not observed. These observations indicate that serotonin may be an important neurochemical mediator of BBB permeability in heat stress.

Brain Edema Formation in Heat Stress

Increased permeability of the BBB is often accompanied with vasogenic edema formation in many pathological conditions. In heat stress, breakdown of the BBB permeability to protein tracers due to increased serotonin levels in plasma and brain is also associated with vasogenic edema formation. This is evident from our studies on the measurement of brain water content, a reliable indicator of edema formation in heat stress. Thus the brain water content showed a significant increase by about 3–4% compared to the control group (Sharma and Cervós-Navarro, 1990). This increase was found only in the experimental animals showing a breakdown of the BBB permeability to proteins (Figure 24.1). Thus the vasogenic edema formation was almost absent in rats exposed to low magnitude of heat of short duration (1 h and 2 h period), or exposed to 4 h heat under anaesthesia. Similarly the brain edema formation was not seen in animals who were made adapted to heat by repeated chronic exposure to mild heat stress (Figure 24.1). These observations strongly suggest that heat stress has the capacity to induce brain edema formation probably via a breakdown of the BBB to proteins.

Cell Injury in Heat Stress

We initiated a series of experiments to study the molecular mechanisms of brain injury at light and electron microscopy using various immunohistochemical markers to identify changes in the neuronal, glial and myelin components of the CNS following heat stress. Using light microscopy we observed profound cell changes in the CNS of rats exposed to 4 h heat stress (Sharma and Cervós-Navarro, 1990; Sharma *et al.*, 1991a). Nerve cells using Nissl stain in many parts of the brain showed that most neurons were shrunken and chromatolytic changes were present in the neuronal cytoplasm (Sharma *et al.*, 1991a). Degeneration of Nissl substance is a very common finding. Many neurons were detached from the neuropil and in most of them a very condensed nucleolus could be found located eccentric within the nuclear cytoplasm. A general sponginess of the neuropil with edematous expansion is quite frequent in various brain regions of the heat stressed rats (Figure 24.2a,b). These nerve cell changes appeared most frequent in the cerebral cortex, hippocampus, cerebellum, thalamus, hypothalamus, reticular formation, pons, medulla and the spinal cord. However, a regional variation seemed to exist. Thus, hippocampus CA-4 subfield showed the most pronounced degenerative changes compared to other regions of the hippocampus. The Purkinje cells showed the more marked nerve cell distortion compared to the granule cells in the cerebellum (Sharma *et al.*, 1991a). These observations suggest that heat has the capacity to induce specific and selective cell damage in the CNS, the basic mechanisms of such a selectivity is not known.

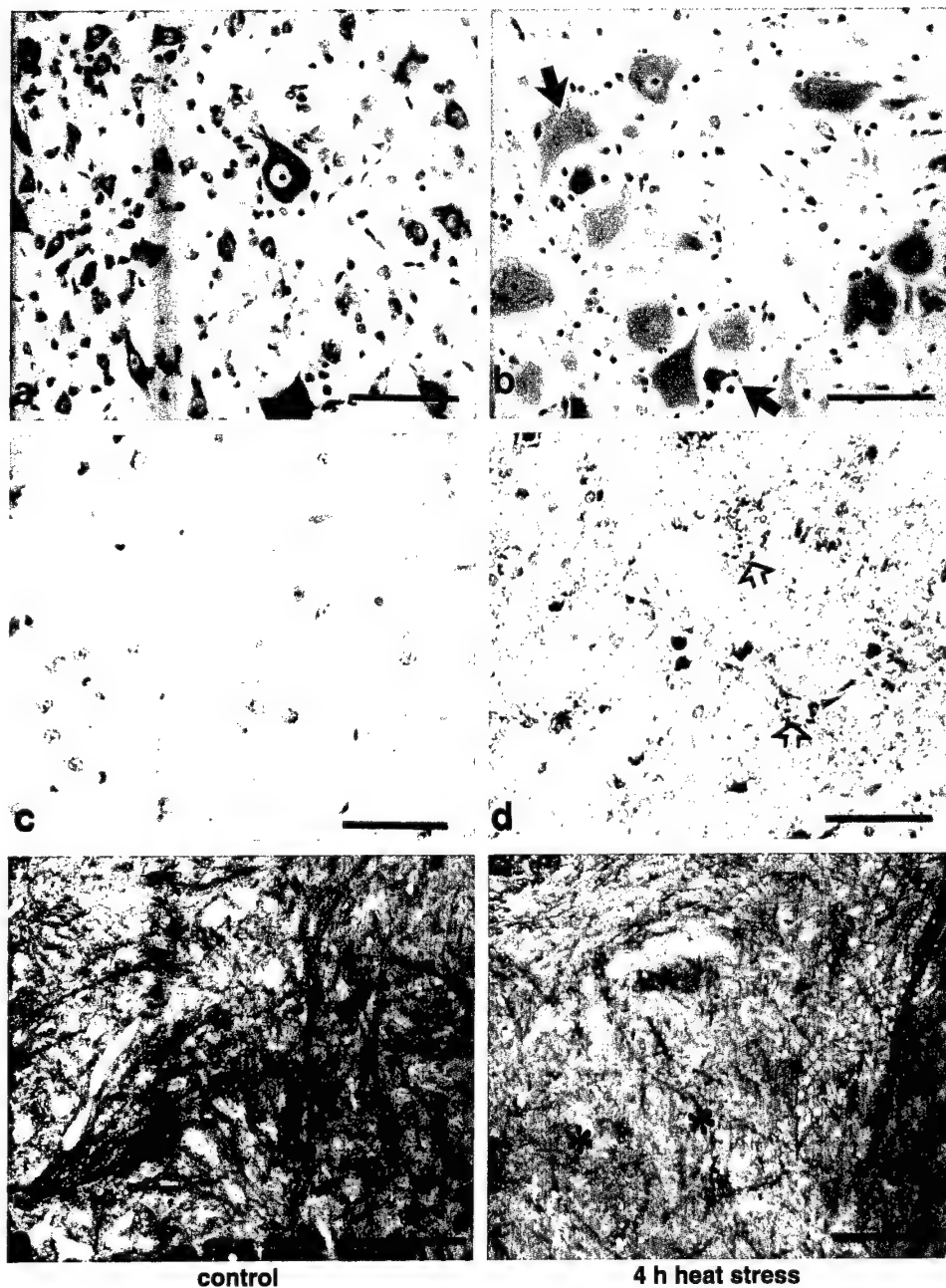


Figure 24.2 Neuronal, glial and myelin changes in the heat stressed rats (right) compared to controls (left). Damaged and distorted neurons with degeneration of Nissl substance in the brain stem region of a heat exposed rat (b) compared to healthy neurons from a normal rat (a). Vacuolation in the cytoplasm, disintegration of cell nucleus and edema around neurons (filled arrows) are quite frequent in the heat stressed rat. Upregulation of glial fibrillary acidic protein (GFAP) immunoreactivity (a marker of astrocytes) seen as brown reaction product is quite extensive in the edematous regions from the brain stem (open arrows) of a heat stressed rat (d). In normal rat GFAP immunoreactivity can occasionally be seen around the extending branches of the star shaped normal astrocytes (c). In the normal spinal cord, immunostaining of myelin basic protein (MBP) is quite intense in many nerve fibres (e) on the other hand in heat stressed rat (f) degeneration of MBP immunostaining along with loss of myelinated fibres is a common feature (*). Bars a, d = 50 μm, b, c = 25 μm, e, f = 30 μm.

We examined the response of glial cells in heat stress using glial fibrillary acidic protein (GFAP) immunoreactivity, a specific marker for astrocytes (Sharma *et al.*, 1992a). The results showed various brain regions exhibiting distorted nerve cells are accompanied with pronounced increase in GFAP immunoreactivity (Figure 24.2c,d). Most profound upregulation of GFAP was seen in the perivascular astrocytes demonstrating a neuronal vessel wall interaction. These observations suggest that astrocytes are one of the sensitive indicators of thermal injury.

Demyelination or vesiculation of myelin occurs following trauma and many other neurodegenerative diseases. However, there are no previous studies regarding alteration in myelin function following heat injury in the CNS. We used immunohistochemistry of myelin basic protein (MBP) to assess myelin damage in heat stress (Sharma *et al.*, 1991a). Our results showed a significant degradation of MBP in many brain regions following heat stress (Figure 24.2e,f). The most striking changes in MBP immunoreactivity is apparent in the reticular formation, pons, medulla and the spinal cord. This finding indicates that myelin sheaths are very sensitive to hyperthermia. Upregulation of HSP 72 kD immunostaining was also found in several brain regions of heat stressed rats exhibiting cell damage. This upregulation of HSP was not found in urethane anaesthetised rats indicating that upregulation of stress proteins following heat exposure is related with cell injury. Our light microscopy results of profound cell damage and edema were further confirmed by electron microscopy. Thus many dark and distorted neurons were present in various regions of the brain of heat stressed rats (Sharma *et al.*, 1991a, 1994, 1996b,c). Edematous swelling of neurons, glial cells and vesiculation of myelin sheaths are evident at ultrastructure level (Figure 24.3). Damage of post synaptic dendrites, membrane disruption and vacuolation are quite common in many parts of the brain.

Pharmacotherapy of BBB in Heat Stress and Brain Injury

The probable mechanisms underlying cell changes following heat stress was examined using various pharmacological agents which can influence BBB permeability in heat stress and thus may interfere with edema formation and/or cell changes. Our observations show that pretreatment with p-chlorophenylalanine (p-CPA), a potent inhibitor of serotonin synthesis or ketanserin, a powerful antagonist of serotonin₂ receptors, prior to heat stress significantly reduced the breakdown of the BBB permeability and edema formation (Figure 24.4). In these rats sign of cell damage in the CNS were absent (Sharma and Cervós-Navarro, 1990; Sharma *et al.*, 1994, 1996b). This indicates that the breakdown of the BBB permeability in heat stress is strongly related with edema formation and cell changes and serotonin appears to play an important role in the pathophysiology of heat stress. However, *in vivo* situations no single chemical compound is responsible for all the observed changes. Thus it appears that many other neurochemical mediators such as prostaglandins, opioids and catecholamines are also involved. Prostaglandins (PGs) are considered as first mediator of stress and involvement of PGs in the mechanisms of thermoregulation and fever are well known. Therefore we inhibited the PG synthesis with indomethacin prior to heat stress and examined the BBB permeability and cell

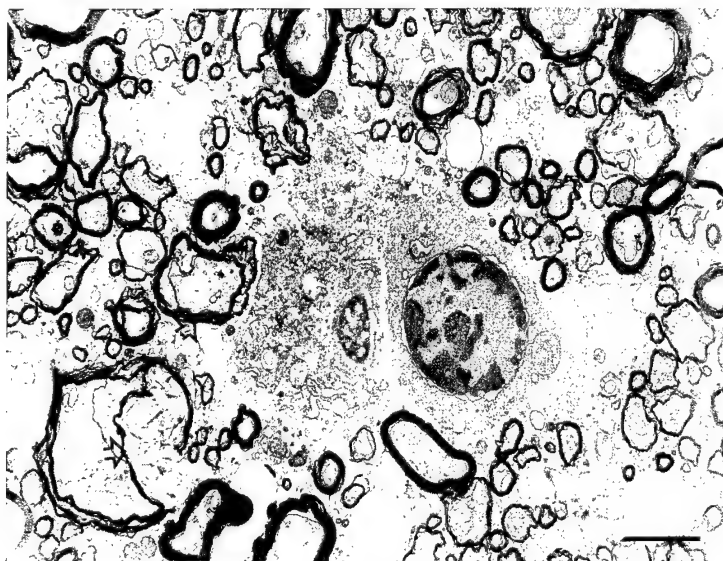
4 h heat stress 38 °C**hippocampus****brain stem**

Figure 24.3 Low power transmission electron micrograph of hippocampus CA-4 sector (above) and brain stem reticular formation (below) from one conscious rat exposed to heat stress. In the hippocampus, one completely collapsed microvessel is present in the middle containing lanthanum (dark black particles) within the lumen. Perivascular edema and synaptic damage (*), the other prominent features of structural changes are quite common. In the brain stem degenerating nerve cells are evident. Signs of membrane damage, vacuolation and edema around the nerve cells are common. Damage of several myelinated nerve fibres and vesiculation of myelin (open arrows) are apparent. Bar = 1 μ m.

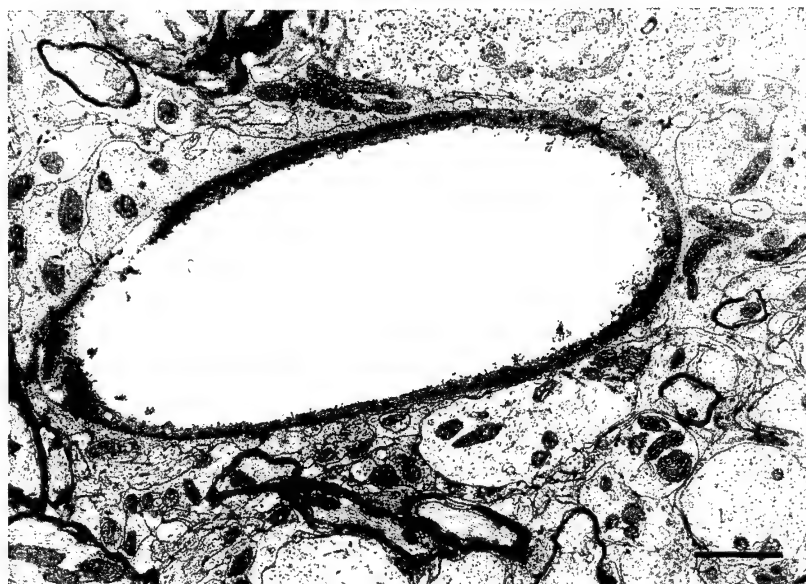
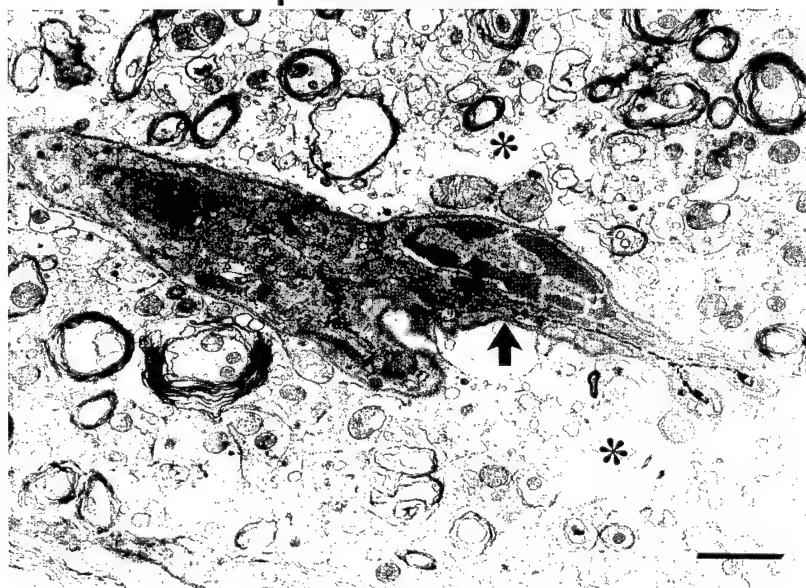
4 h heat stress 38 °C**p-CPA treated****6-OHDA treated**

Figure 24.4 Low power transmission electron micrograph from the brain stem reticular formation of one conscious rat exposed to heat stress pretreated with p-CPA (above) and one rat pretreated with 6-OHDA (below). p-CPA markedly reduced the ultrastructural changes in heat stressed rat whereas 6-OHDA was ineffective in reducing cell damage. Perivascular edema (filled arrow), synaptic damage (*) and vesiculation of myelin is quite prominent in 6-OHDA treated rat, a feature which is absent in p-CPA treated animal. Bar = 2 μ m.

changes (Sharma and Dey, 1987). Our results showed that inhibition of PGs prior to heat stress significantly attenuated BBB permeability, and edema formation. This treatment also reduced the occurrence of brain damage indicating an involvement of PGs in the pathophysiological mechanisms of heat stress.

Endogenous opioids play an important role in the mechanisms of thermoregulation and fever. Opioids are also involved in the brain pathology caused by traumatic injury of the CNS (Sharma *et al.*, 1996c). However contribution of opioids in breakdown of the BBB permeability in heat stress is not yet known. Thus, we examined the involvement of opioids in the pathophysiology of heat stress by using a multiple opioid receptor antagonist naloxone (Sharma *et al.*, 1996). We used a high dose of this compound in order to block both the m-, d- and also the k-opioid receptors. Our results show that pretreatment with naloxone in high doses attenuated the breakdown of the BBB permeability and reduced the edema formation and cell changes indicating a positive role of opioids in the BBB and brain pathology caused by heat stress (Sharma *et al.*, 1996c). Apart from serotonin, prostaglandin and opioids catecholamines are also involved in the basic mechanisms of stress and thermoregulation (Selye, 1976). Altered levels of catecholamines are found in various CNS diseases, however, its role in the pathophysiology of heat stress is still unknown. Therefore, we used a selective neurotoxin, 6-hydroxydopamine (6-OHDA) to degenerate both the peripheral and central catecholaminergic neurons with intravenous and intracerebroventricular administration of the compound, respectively (Sharma, 1982). The result of this study showed that degeneration of catecholaminergic nerve fibres and terminals prior to heat stress markedly aggravated the breakdown of the BBB permeability. In these rats, the edema formation and cell changes were much more pronounced (Figure 24.4). These observations suggest that catecholamines are playing an inhibitory role in the pathological mechanisms of hyperthermia and strongly supports the idea that breakdown of the BBB permeability is instrumental in precipitating morphological abnormalities in the CNS.

CONCLUSION

Taken together, it appears that drugs which has the capacity to attenuate the BBB breakdown in heat stress have significantly reduced or prevented the cell changes occurring in the CNS whereas, the drug which did not protect the BBB leakage was not able to reduce adverse cell reaction. These experimental observations in heat stress are in line with the idea that the BBB can be regarded as a gateway to various brain diseases leading to brain pathology. This breakdown of the BBB in brain diseases appears to be mediated by several neurochemicals.

FUTURE DIRECTION

The future goal of our laboratory is to map various neurochemicals, their receptors and second messenger system in different brain regions using immunohistochemical and/or *in situ* hybridisation technique in relation to the BBB breakdown in heat

stress. These investigations will further expand our knowledge to gain a deep insight into the basic molecular mechanisms of hyperthermic brain injury.

Acknowledgements

Due to space limitation only key references are included. Financial support for this investigation is received by The University Grants Commission, New Delhi, Indian Council of Medical Research, New Delhi, India; Alexander von Humboldt Foundation, Bonn, Germany; Swedish Medical Research Council project nos. 2710, 9459, 10954; Göran Gustafsson Foundation, Stockholm, Sweden. We are grateful to R. Esparza, Elisabeth Scherer, Franziska Drum, Katja Deparade, Hana Plückhan (Berlin, Germany); M. Siddiqui, Aftab Ahmed, A.N. Pandey, R.C. Gupta (Varanasi, India); Kärstin Flink, Ingmarie Olsson, Gunilla Tibbling, Madelline Thörnwall, Madeleine Järild (Uppsala, Sweden) for technical assistance; Frank Bittkowski for photography and William Schannong for assistance in computer work. The secretarial assistance of Angela Ludwig, Katherin Kern, Gunilla Åberg, GunBritt Lind, Agneta Bergstörn and Aruna Misra is highly appreciated.

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25 Stress-Induced Central Nervous System Penetration by Non-Invasive Attenuated Encephalitis Viruses

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The following studies explored the effects of physical (cold), social (isolation) and pharmacological (corticosterone) stress on attenuated arboviruses penetration into the CNS leading to encephalitis. As experimental models, we used two neurovirulent viruses that lack neuroinvasive capacity; WN-25, a variant of the West Nile virus, a neuroadapted Sindbis virus (SVN) and a neuroinvasive avirulent variant of Semliki Forest virus (SFV-A7). Exposure of WN-25 and SVN infected (i.p.) mice to different stress paradigms such as cold, isolation or corticosterone (cs) injection resulted in the penetration of the virus into the CNS, causing encephalitis and death. Whereas no effect was observed in non-stressed infected mice. The WN-25 virus extracted from the brain of the moribund mice (10^8 PFU) showed a change in its neuroinvasive properties, raising the possibility of stress-enhanced proliferation and a selective process leading to reversion to neurovirulence. In contrast, brain SVN virus levels were found to be more than 10^7 PFU in infected moribund mice exposed to stress. In this case, no changes were detected in the neuroinvasive properties of recovered progeny virus extracted from the moribund mice. We conclude that stress induces a breach in the blood-brain barrier and enables the penetration of attenuated viruses (WN-25 and SVN) into the CNS.

INTRODUCTION

Stress has been defined by Selye (1950) as a nonspecific response to internal and external stimuli that induce hormonal changes which lead to suppression of the immune system. Evidence has been provided demonstrating connections between stress and the immune system (Riley, 1981; Dantzer and Kelley, 1989), and between the immune system and the central nervous system (CNS) (Rouabhia *et al.*, 1991). The interaction of the immune system with the brain play a significant role in the outcome of infectious diseases. The effects of stress on the immune system in human and animal studies are most commonly suppressive, with changing production and activity of a wide range of immune system components (Peterson *et al.*, 1991; Cohen and Williamson, 1991; Goetzl and Sreedharan, 1992). A variety of stress paradigms have been shown to exacerbate the effects of several infectious agents, including

herpes simplex virus (Rasmussen *et al.*, 1957), influenza virus (Feng *et al.*, 1991; Hermann *et al.*, 1994) and encephalitic viruses (Ben-Nathan *et al.*, 1989, 1991; Ben-Nathan and Feuerstein, 1990). Viruses are known to flourish in the stressed host, with increased morbidity and mortality (Sheridan *et al.*, 1994; Friedman *et al.*, 1970).

Glucocorticoids (GC) are major mediators in the reaction to stress and in stress-induced immunosuppression (Riley, 1981; Dantzer and Kelley, 1989; Khanshari *et al.*, 1990). Such immunosuppression is particularly evident with viral infections (Gatmaitan *et al.*, 1970). The administration of GC during viral infection leads to higher viral titers and increased morbidity and mortality (Riley, 1981; Ben-Nathan *et al.*, 1989). The effect is similar to that of stress on viral infections (Riley, 1981; Ben-Nathan *et al.*, 1996). Furthermore, it was demonstrated that viral infections cause a stress reaction with elevation of endogenous GC levels (Blalock, 1987; Smith *et al.*, 1982), involution of lymphoid organs and generalized immunosuppression (Riley, 1981; Ben-Nathan and Feuerstein, 1990). Viral infections, produced by retroviruses such as HIV, Feline or Friend leukemia virus are associated with prolonged immunosuppression, i.e., down regulation of the immune response. The effects of stress on susceptibility of animals to bacterial and viral infections were recently reviewed by Sheridan *et al.* (1994), Cohen and Williamson (1991) and Dantzer and Kelley (1989).

The following studies explored the effects of physical (cold), social (isolation) and pharmacological (corticosterone) stress situations on the course of viral encephalitis. As an experimental model, we used two neurovirulent viruses that lack neuroinvasive capacity (Halevy *et al.*, 1994; Lustig *et al.*, 1992a,b); WN-25 and SVN. An avirulent variant of Semliki Forest virus, SFV-A7 (Fazakerley *et al.*, 1993), that has neuroinvasive capacity has also been used. These variant strains differ from the parent strains (see Table 25.1) by their inability to kill mice by intraperitoneal injection (i.p.) under normal conditions. Therefore, we chose these viruses as a suitable model for studying the effect of stress on the course of viral infections.

Table 25.1 Neurovirulence and neuroinvasiveness of the West-Nile, Sindbis and Semliki-Forest viruses

<i>Virus strain</i>	<i>PFU/ml</i>	<i>i.c. LD₅₀</i>	<i>i.p. LD₅₀</i>
WNV	1.0×10^7	1.6×10^7	1.3×10^7
WN-25	6.0×10^6	1.5×10^6	<32
SVNI	1.5×10^8	5.7×10^7	9.1×10^5
SVN	1.1×10^8	5.8×10^7	<32
SFV	2.1×10^8	1.5×10^8	5.3×10^7
SFV-A7	1.4×10^8	<32	<32

WNV — West-Nile virus, wild type. WN-25 — Attenuated variant of WNV. SVNI — Neurovirulent invasive strain of Sindbis virus. SVN — Neuro-adapted noninvasive Sindbis strain. SFV — Semliki Forest virus, wild type. SFV-A7 — avirulent strain of SFV. Characteristics defined in 4 weeks old mice.

RESULTS AND DISCUSSION

Measurements of Stress Markers

The effect of stress on lymphoid organs weight

Stress is known to induce involution of lymphoid organs such as thymus, spleen and lymph nodes. This involution serves as a common marker for assessing immunosuppressive effects of stress. To study the general effect of the stress paradigms used on mice, spleen and thymus weights were measured after 7 days of exposure to stress. Cold (physical) and isolation (social) stress both reduced spleen and thymus weight. SVN inoculation of non-stressed mice induced a non significant reduction of lymphoid organs weight. However, the combined effect of virus inoculation and stress administration induced a significant weight reduction (Figure 25.1).

Corticosterone level in serum of mice exposed to cold stress

Corticosterone levels in serum of mice exposed to cold stress treatment were measured. Cold stress caused an increase in CS levels, from a basal level of 97.2 ± 7 ng/ml to a sera level of 482.6 ± 36 ng/ml after 1 h and to 463.2 ± 25.4 ng/ml after 2 h ($p < 0.01$). Therefore, we used CS administration as a pharmacological mean to simulate a general stress treatment.

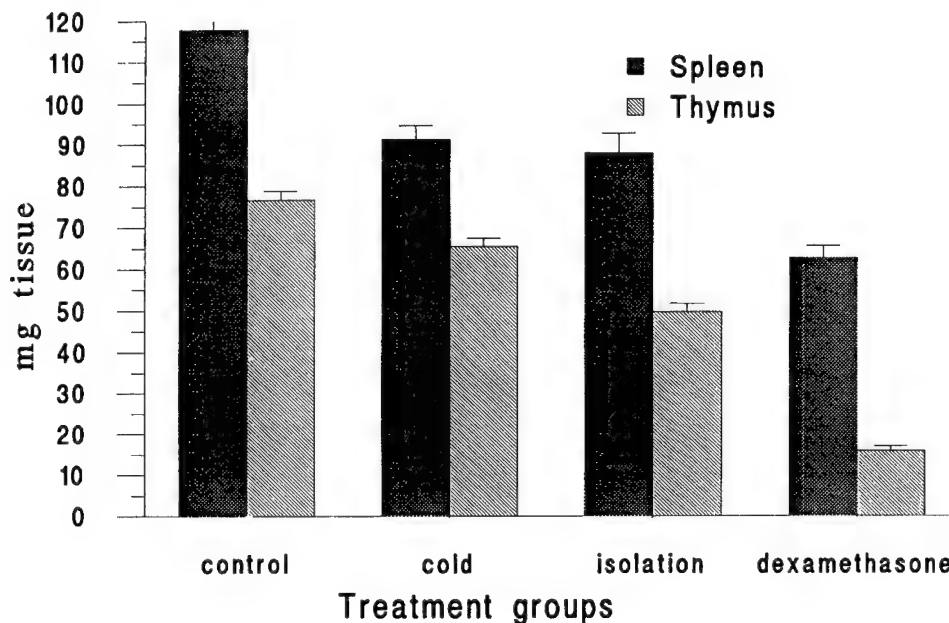


Figure 25.1 Effect of stress on splenic and thymic involution in mice 7 days after exposure to cold, isolation or dexamethasone treatment (2 mg/kg i.m. for 3 days). Cold stress: mice were placed for 5 min in 3 cm deep cold water ($1 \pm 0.5^\circ\text{C}$) daily for 7 days. $N=8$, $P < 0.05$ as compared to non-stressed group.

Stress and Viral Infection

A. Characterization of the attenuated viruses

The various viruses described in Table 1 differ in their neurovirulent and neuroinvasive properties. Wild type WNV, (genus flavivirus) exhibits both neuroinvasive and neurovirulent traits, killing mice when injected either i.c. or peripherally. Its attenuated counterpart — WN-25 — is a noninvasive neurovirulent virus, causing death only when injected i.c., but not when administered peripherally. A pair of Sindbis viruses (genus alphavirus), showing similar properties to the WN pair, were obtained from a pool of avirulent Sindbis viruses. Both viruses — SVNI and SVN — will kill mice when injected i.c., but only SVNI has such effect when injected peripherally. An additional pair of alphaviruses — SFV and SFV-A7 — comprises a neuroinvasive neurovirulent wild type and its neuroinvasive avirulent counterpart.

B. Effect of various stress paradigms on mortality and brain virus levels

The purpose of these experiments was to determine the effect of various stress stimuli on mortality of mice inoculated with attenuated arboviruses by the i.p. route. The mice were exposed to cold treatment, isolation or corticosterone administration. As shown in Table 25.2 the stress paradigms induced mortality rates of 65%, 80% and 50% (WN-25) and 75%, 78% and 58% (SVN) in cold, isolation and CS respectively. No mortality was seen in non-stressed i.p. inoculated mice (Table 25.2). Similarly, those same stressors can induce SFV-A7 to cause a fatal infection. SFV-A7 strain caused encephalitis and death in stressed mice, with no death occurring among the non-stressed SFV-A7 inoculated mice (Table 25.2). This susceptibility of stressed mice to viral infection could be explained by the finding that stress induced the suppression of macrophage and T-lymphocyte activities (Bernton *et al.*, 1988) and decreased the natural killer cell cytotoxicity (Shavit *et al.*, 1984). Therefore, stress effect can maintain elevated virus levels are maintained for longer periods, facilitating

Table 25.2 Effect of stress treatments on mortality and brain virus levels in mice inoculated with attenuated arboviruses

Treatment group	Mortality and brain-virus levels (log ₁₀ PFU)					
	WN-25		SVN		SFV-A7	
	D/T	PFU/brain	D/T	PFU/brain	D/T	PFU/brain
Control	0/18	<2	0/12	<2	0/12	5.8±0.4
Cold	13/20	8.5±0.4	15/20	7.3±0.2	6/10	8.1±0.2
Isolation	16/20	7.8±0.3	14/18	7.1±0.2	7/12	8.0±0.3
Corticosterone	6/12	7.5±0.4	7/12	7.0±0.2	10/18	7.4±0.2

D/T — Dead from total inoculated mice. Corticosterone, 10 mg/kg injected i.v. one day after virus inoculation.

Isolation — one mouse per cage. Cold stress treatment (5 min a day at 1±0.5°C) was administered on the day of virus inoculation and afterwards. Six mice were tested in each group — all mice were sacrificed on day 7.

the penetration of viruses into the CNS (Ben-Nathan *et al.*, 1996). Although the overt effect of stress on mice inoculated with the attenuated viruses seem to be the induction of encephalitis and death, the presence of the viruses in the brain illustrates the effect in the CNS concentration. The virus levels in brains of moribund stressed mice were determined and the results are presented in Table 25.2.

The brain virus levels in control mice inoculated with WN-25 or SVN showed titers of less than $2 \log_{10}$ PFU; whereas the virus titers in the brain of cold, isolation or cs treated mice were 8.5, 7.8 and 7.5 (\log_{10} PFU) for WN-25 and 7.3, 7.1 and 7.0 (\log_{10} PFU) for SVN respectively. In stressed mice, these viruses (WN-25 and SVN), both lacking neuroinvasive properties invade the brain and the virus levels were found to be more than 10^6 PFU as compared to control inoculated mice. In SFV-A7 inoculated mice, the brain virus titers were 5.8, 8.1, 8.0 and 7.4 \log_{10} PFU in control, cold treatment, isolation, or cs administration, respectively (Table 25.2).

C. Transfer of stress effect on neuroinvasion by sera of stressed mice

To explore the effect of cold stress on attenuated virus neuroinvasion, an attempt was made to induce viral penetration by transfusion of sera from cold-stressed mice. Mice were bled 2 hours after one exposure to cold stress and the sera obtained were assayed for CS content and were injected in combination with the attenuated virus. WN-25 virus was diluted in the donor sera to a final dilution of 2×10^5 PFU/ml and 0.2 ml were injected i.v. into naive mice. The results shown in Table 25.3, demonstrate the capability of donor sera from stressed mice to induce viral penetration into the brain. As can be seen from the results described in Table 25.3, donor sera obtained from stressed mice 2 h after cold exposure, could induce encephalitis of the attenuated virus, with a mortality rate of about 78%. The different treatments, cold, CS and passive transfer of sera can induce encephalitis and death of 50%, 44% and 78%, respectively.

Table 25.3 The effect of cold stress or serum from cold exposure mice on mortality of mice inoculated with WN-25

Treatment group	Mortality	
	D/T	%
WN-25 i.p.	0/8	0
WN-25 i.v.	0/8	0
WN-25 i.v. + Cold *	6/12	50
WN-25 + SD i.v.	15/18	78
WN-25 + CD + CS i.v. **	7/16	44
WN-25 + CD i.v.	0/8	0

* Mice were exposed to cold water, 5 min a day ($1 \pm 0.5^\circ \text{C}$) for 8 days. SD were obtained from CD-1 mice 2 h after cold stress exposure. Corticosterone level in SD was $373.3 \pm 16.6 \text{ ng/ml}$, and 94.5 ± 8.4 in CD (serum from naive mice).

** CS (5000 ng/mouse) was diluted in CD. WN-25 virus was diluted in DS or CD and 0.2 ml were injected i.v. to naive CD-1 mice.

No mortality could be shown with sera obtained from control mice. However, it should be noted that the amount of CS present in the donor sera (100 µg) is only 2–4% of that required for exogenous CS to cause a similar effect.

Our results showed that the neurovirulent non-neuroinvasive viruses can serve as markers for blood–brain-barrier permeability, and aid in studying pathological processes of the brain. Moreover, we suggest the use of these viruses as viable pathogenic indicators for stress in the animal.

D. Stress and changes in virus traits

After intracerebral (i.c.) injection of WN-25 virus into mice, the isolated virus from the brain did not show any change in its virulence. The findings showed that the virus isolated from brains of stressed moribund mice was very virulent, killing mice by i.p. injection of as little as 10 PFU. *In vivo* assay of the virus isolated from stressed mice, the I.P.LD₅₀ increased by 10⁶ indicating an extreme increment in replication and virulence. The data clearly showed that in stressed mice the avirulent WN-25 strain become virulent and reached a titer similar to the original WNV in control mice.

From the results it appears that stress suppressed the immune response resulting in impaired macrophages and lymphocytes function. This leads to enhance viral replication in the blood and peripheral organs, allowing mutational processes and selection of a neuroinvasive strain. This chain of events has not been detected with SVN or SFV-A7.

Table 25.4 is a schematic presentation of the various effects of stress treatments on the outcome of viral infections caused by attenuated viruses. The exacerbating effect of stress was clearly demonstrated with each one of the virus tested. The main effect seen with the noninvasive strains, WN-25 and SVN, was the induction of virus penetration into the CNS. However, comparison of the viruses extracted from the brain of stressed mice with the original strain showed a change in the virulence of the WN virus, but not in the Sindbis strain.

Therefore, stress may indirectly contribute to the selection of a neuroinvasive variant of WN-25 during infection. However a different mechanism, activated by stress treatment, enables SVN penetration into the CNS.

On the other hand, SFV-A7 is a neuroinvasive attenuated virus, which will cause the death of the inoculated stressed mice. In this case stress doesn't mediate the induction of viral penetration. Stress-induced immunosuppression enhanced the proliferation of the virus in the CNS and exacerbate the effect of the infection.

Table 25.4 Effect of stress on viral functions*

<i>Virus tested</i>	<i>Control mice</i>		<i>Stressed mice</i>		
	<i>CNS penetration</i>	<i>Mortality</i>	<i>CNS penetration</i>	<i>Mortality</i>	<i>Change in virulence</i>
SVN	—	—	+	+	—
SFV-A7	+	—	+	+	—
WN-25	—	—	+	+	+

* Tested in 4 week old mice.

The data presented in this manuscript provide evidence for an association between stress and pathogenesis of infectious diseases, stress induced immunosuppression markedly influences the infectious process.

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VI. Perspectives for Drug Discovery

26 Role of Molecular Biology in Strategies to Discover Novel Targets for Drug Development

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Differential gene expression is essential for normal development and a variety of pathological conditions of the central nervous system. It is generally reflected by the number of mRNAs expressed in a given cell at any time point. Changes in relative mRNA levels may have important implications for the development of pathological processes. Therefore, the discovery of differentially expressed genes is critical for understanding the molecular mechanisms involved in normal and pathological states, as well as for providing new insights for the discovery of new molecular targets for pharmacological manipulation and drug development.

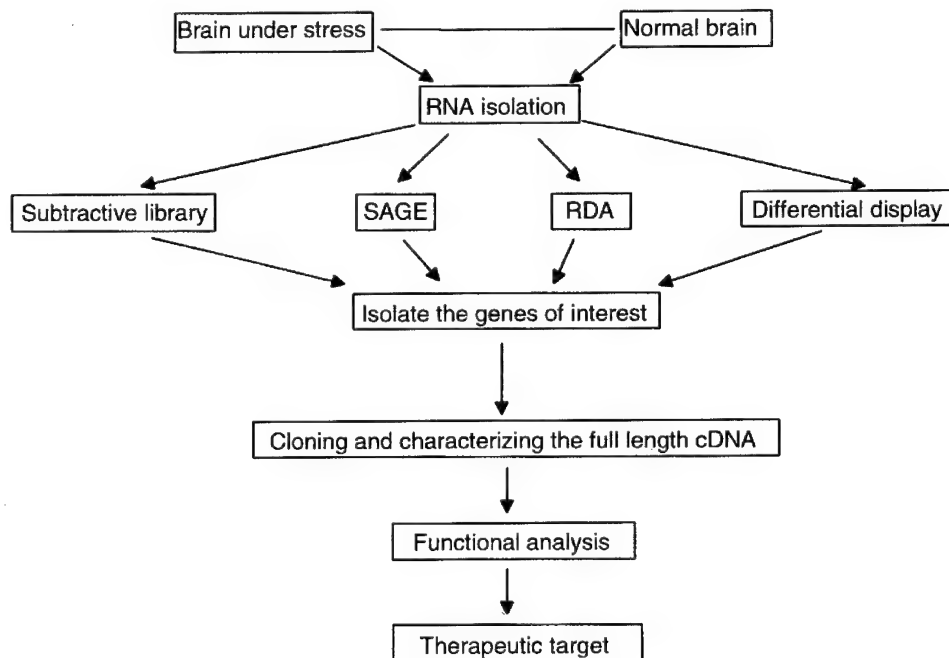
Stress situations represent pathophysiologic conditions that modulate gene expression and protein functions in the brain. For example, the upregulation of heat-shock proteins has been observed in a number of stress situations including brain ischemia and trauma (Massa *et al.*, 1996). However, the overall molecular mechanisms of brain responses to stress are not well understood. One way to better understand the molecular mechanisms associated with brain response to stress is to identify and characterize genes of altered expression.

A number of techniques (e.g., subtractive library screening, differential hybridization, serial analysis of gene expression, representational difference analysis and mRNA differential display) have been developed for novel gene discovery (Table 26.1). Subtractive library screening and differential hybridization are conventional methods for the identification of molecules that differ in abundance between two pools of molecules. Representational difference analysis (RDA) and mRNA differential display are polymerase chain reaction (PCR)-based techniques and are sensitive for the detection of differentially expressed genes. The serial analysis of gene expression (SAGE) method uses small (9 base) nucleotide sequence tags to analyze the genes that are expressed in a particular system and for the discovery of novel genes. In this chapter, we describe overall strategies for the discovery of disease/stress related gene expression (Figure 26.1), with special attention to mRNA differential display technique as an example for a successful application in a particular

Table 26.1 Comparison of the molecular techniques used for novel gene discovery

	<i>Key technique</i>	<i>Applications</i>	<i>Advantages</i>	<i>Disadvantages</i>
Subtractive library	Hybridization-based subtraction	Isolate the differentially expressed genes	Highly reproducible	Relative insensitive; labor intensive
SAGE	Enzyme cleavage, ligation and amplification to create a short sequence tag	Observe the expressed genes	Allows quantitative, simultaneous analysis of a large number of transcripts	Complicated; labor intensive; 9-mer tag is a suboptimal probe
RDA	Subtraction coupled to PCR amplification	Isolate the differentially expressed genes	Very sensitive	Technically difficult and labor intensive
Differential display	Modified RT/PCR	Isolate the differentially expressed genes	Sensitive; allows multiple comparison for up- and/or down-regulated genes	High incidence of false positives; many ESTs are 3'-UTR

Cloning of Stress-Regulated Gene Expression in Brain

**Figure 26.1** Overall strategy for discovery of stress-induced gene expression in brain.

condition of brain injury, i.e., brain ischemia. While the example provided in this chapter is drawn from brain injury by a pathophysiologic condition (stroke), the method is applicable to all tissues and cells and therefore likely to be of great value also in stress research.

STRATEGIES FOR DISCOVERING DISEASE/STRESS RELATED GENE EXPRESSION

Subtractive Libraries

The critical feature of subtractive cDNA library is to remove the common mRNA species in two different tissues or experiment sources (Hedrick *et al.*, 1984). Subtraction may be carried out between two mRNA pools or two cDNA libraries. This method is favorable for the cloning of abundant messages.

Differential Hybridization

Differential hybridization or differential screening (Tedder *et al.*, 1988) is an alternative to the subtractive library cloning. However, the differential hybridization requires only a single library of interest. The cDNA library is plated out and lifted in duplicate. Hybridization probes are generated by reverse transcription in the presence of ^{32}P - α -dNTP and either oligo-dT or random primers using two mRNA pools (i.e., experimentals and controls). The differentially expressed genes are identified by using these two probes with the duplicate lifts.

Serial Analysis of Gene Expression (SAGE)

SAGE is a recently developed method for the investigation of the expressed genes (Velculescu *et al.*, 1995). This technique is based upon two principles: (1) a short nucleotide sequence tag containing sufficient information to uniquely identify a transcript; (2) concatenation of short sequence tags allowing the efficient analysis of a large number of transcripts. Therefore, SAGE allows the quantitative and simultaneous analysis of a large number of transcripts.

Representational Difference Analysis (RDA)

RDA is a process of subtraction coupled to PCR amplification. It was originally developed for the use of genomic DNA to isolate genomic differences between two complex genomes (Lisitsyn *et al.*, 1993). The genomic RDA has been adapted for use with cDNA to identify differences in mRNA expression (Hubank and Schatz, 1994). RDA is a very sensitive technique for the detection of differentially expressed genes, but it is technically difficult, time consuming, and labor intensive.

Differential Display

Initially introduced by Liang and Pardee (1992), mRNA differential display is a PCR-based technique to isolate genes of interest in a variety of biological systems;

it has become increasingly popular due to its technical simplicity (Liang and Pardee, 1995). The method of mRNA differential display consists of two basic steps: (1) reverse transcription (RT) using a set of 3'-anchored primers, $T_{12}MN$ ($M=G, A$ or C ; $N=G, A, T$ or C), and (2) PCR amplification of cDNA fragments using arbitrary 10-mer primers (upstream) and anchored downstream primers. One critical feature of this technique is to display most of the mRNA population on a sequencing gel after PCR.

A similar technique referred to as RNA fingerprinting by arbitrary primed PCR (RAP-PCR) was introduced to identify differentially expressed genes (Welsh *et al.*, 1992). The use of longer primers (18–20 mer for both RT and PCR) in the RAP-PCR method increased the reproducibility of the band display pattern but displayed fewer bands than the mRNA differential display.

Differential display PCR

For the RT reaction, total cellular RNA is reverse-transcribed to yield the first strand cDNA primed with $T_{12}MN$ oligonucleotides ($T_{12}MN$ where $M=G, A, C$ and $N=G, A, T$, or C). This RT reaction enables all the mRNA species having a poly(A) tail to be reverse-transcribed. Typically, this reverse transcription reaction is divided into 4 subgroups each using a different $T_{12}MN$ primer with G, A, T or C at the last base of the 3'-end.

Amplification is carried out using an upstream arbitrary primer and a downstream anchored primer in the presence of a radioactive nucleotide. The upstream primer typically consists of 10 bases in length containing approximately 50% of GC content. In addition, a relatively low annealing temperature (42°C) is used to allow some base mismatches to increase the number of mRNA species to be amplified. The amplified cDNA fragments are resolved by electrophoresis and subjected to autoradiographic analysis.

Band recovery and confirmation of the differentially expressed genes

Following mRNA differential display, the band of interest is excised from the dried sequencing gel, isolated by extraction procedures, and reamplified using the same sets of primers as in the original PCR. The recovered DNA band can serve as a probe to confirm mRNA expression by means of Northern blot analysis, and/or can be subcloned into a vector for further analysis.

Confirmation of gene expression is one of the critical steps following mRNA differential display, in as much as a large number of false positive bands may be present. A variety of methods have been utilized in different laboratories to reduce false positives; the most commonly used method is Northern blot analysis. In addition, dot blot, quantitative RT/PCR, ribonuclease protection assays, and other methods have also been used.

Identification of the differentially expressed genes

It is critical to identify the genes discovered by mRNA differential display. This step relies on the DNA sequencing analysis of the recovered DNA band. Because the

primers used for differential display are short and cannot be used successfully for direct sequencing by standard protocols, the differential displayed DNA fragments are typically subcloned into a vector prior to sequencing analysis. Recently, direct sequencing of differential display PCR products become feasible based upon the use of elongated primers for direct differential display (Zhao *et al.*, 1995; Diachenko *et al.*, 1996) or during the reamplification following original differential display method (Wang and Feuerstein, 1995).

Based upon the sequence information, the identity of the differentially expressed genes can be determined by searching a computer database (e.g., GenBank). If the sequence represents an unknown sequence, a cDNA library can be screened using this DNA as a probe to obtain the full length cDNA clone.

Recently, significant improvements and modifications have been made to the method as originally described (Liang and Pardee, 1992) in order to overcome some of the existing problems in this technique. For example, to reduce the frequency of false positives, the importance of DNA-free RNA samples and multiple displays of samples have been emphasized (Liang *et al.*, 1993); the use of longer primers, e.g., 18–20 mers, as used for RNA-fingerprinting (Welsh *et al.*, 1992), not only increases the reproducibility of differential display but also allows direct sequencing after PCR amplification (Zhao *et al.*, 1995; Diachenko *et al.*, 1996); to avoid the hazardous nature of ^{35}S as a radiolabel for differential display, either ^{32}P or ^{33}P is recommended as an alternative label (Trentmann *et al.*, 1995; Tokuyaman and Takeda, 1995).

DISCOVERY OF ADRENOMEDULIN IN STROKE USING DIFFERENTIAL DISPLAY

Identification of Ischemia-Induced Gene Expression

As shown in Figure 26.2A, mRNA differential was used to investigate the cerebral focal ischemia-induced expression and a band designated as PMCAO-9 was identified. The upregulation of this gene expression in ischemic cortex was confirmed using Northern analysis (Figure 26.2B). Thereafter, PMCAO-9 was subcloned into a pCRII vector and subjected for DNA sequencing analysis. Full length cDNA was cloned using PMCAO-9 cDNA probe, which was identified as the adrenomedulin (AM) gene (Wang *et al.*, 1995).

Temporal Expression of AM mRNA in Rat Ischemic Cortex After Middle Cerebral Artery Occlusion (MCAO)

The extended time course of AM mRNA expression was examined in the ipsilateral (ischemic) and contralateral (nonischemic) cortical samples after MCAO (Figure 26.3A). Quantitative Northern blot data ($n=4$), after normalizing to a rpL32 probe, are illustrated graphically in Figure 26.3B. A very low level of AM mRNA was detected in normal brain or in sham-operated cortical samples. The AM mRNA expression was induced significantly in the ischemic cortex 3 h after MCAO (17.4-fold increase compared to sham), reached its peak expression at 6 h (21.7-fold

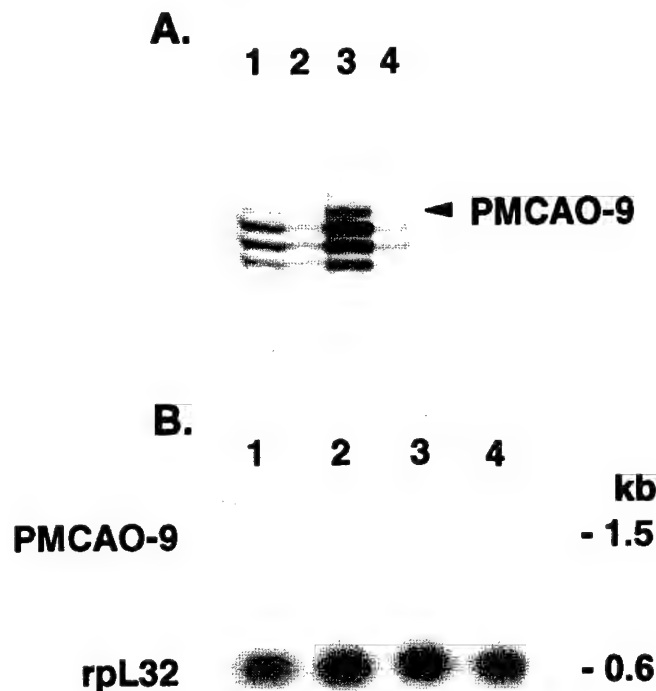


Figure 26.2 Identification of altered gene expression in rat ischemic cortex following MCAO by mRNA differential display. **A.** Analysis of PCR products from mRNA differential display. RNA samples were isolated from 2 and 12 h after MCAO in rat and reverse transcribed with T₁₂NA primers (N=G, A, T, or C). PCR amplification was performed using a 5' decamer arbitrary primer (5'-GACCGCTTGT-3') and a 3' T₁₂NA primer in the presence of [³⁵S]dATP. The PCR reaction was carried out for 40 cycles as follows: 94°C 30 seconds for denaturing, 40°C 2 min for annealing, 72°C 30 s for extension, followed by 1 cycle for extension at 70°C for 10 min. The amplified products were resolved in an 8 M urea, 6% polyacrylamide DNA sequencing gel and analyzed by autoradiography. Lane 1, 2 h ischemic, lane 2, 2 h non-ischemic, lane 3, 12 h ischemic and lane 4, 12 h non-ischemic. The candidate gene indicated with an arrowhead (PMCAO-9) was further analyzed. **B.** Northern analysis of the PMCAO-9 gene expression in rat ischemic and non-ischemic cortex at 2 and 12 h post-PMCAO. Poly(A) RNA (2 µg/lane) was resolved by electrophoresis with the same loading order as in A, transferred to a nylon membrane, and hybridized to PMCAO-9 and ribosomal protein L32 (rpL32; loading control) cDNA probes sequentially.

increase), and remained elevated for at least 15 days (9.6-fold increase) following MCAO (Figure 26.3).

The upregulation of AM protein after focal brain ischemia was also demonstrated and the cellular source of AM has been localized in the neuronal filaments (Wang *et al.*, 1995).

Functional Analysis of AM on MCAO Injury

To investigate the biological function of the induced expression of AM in the ischemic cortex, we administered AM intracerebroventricularly (ICV) in the brain

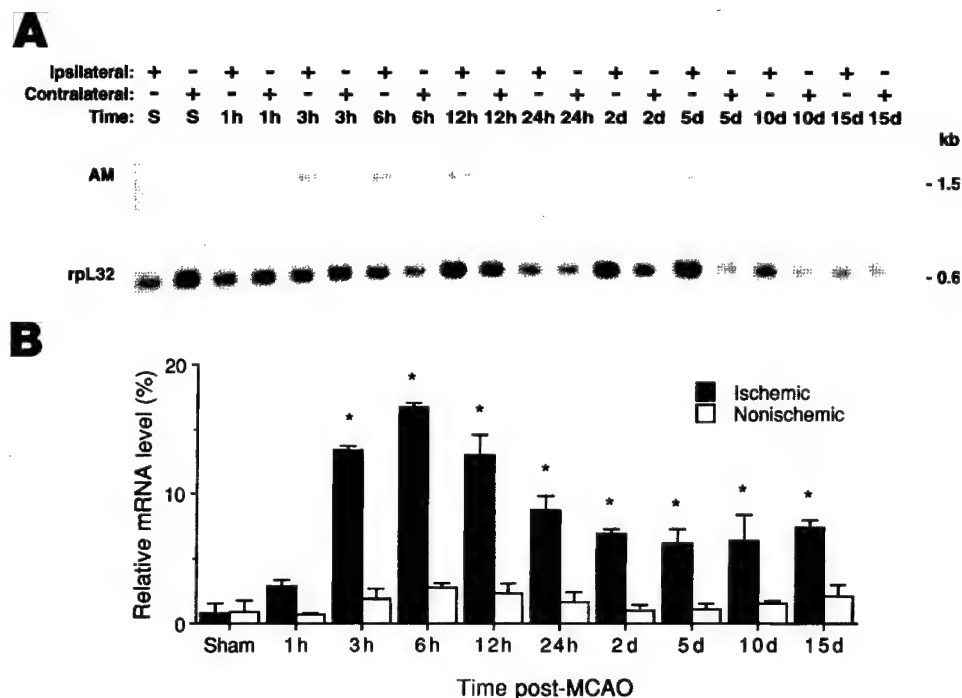


Figure 26.3 Time-course study of adrenomedullin mRNA induction in rat ischemic cortex following MCAO. A. A representative Northern blot for AM and rpL32 probes to the samples isolated at various time points and conditions from rats subjected to MCAO. Total cellular RNA (40 µg/lane) was used for this analysis. Ipsilateral and contralateral cortex samples (denoted by +) from individual rats following sham surgery (S; 12 h sacrifice) or 1, 3, 6, 12, 24 h, and 2, 5, 10, 15 days of MCAO are depicted. B. Quantitative Northern blot data for AM mRNA expression following focal brain ischemic injury. The data were analyzed using PhosphorImager and displayed graphically in a sum of 100% after being normalized to the values of the rpL32 mRNA signals. Data are presented as the mean values \pm standard error of four separate experiments in spontaneously hypertensive rats ($n=4$) for each time point, and analyzed by one-way ANOVA followed by Bonferroni-adjusted post-hoc t -test. * $p < 0.05$, vs sham samples.

and examined its effect on MCAO injury. The results (Figure 26.4) indicate that ICV, but not systemic, AM administration at high dose (8 nM), prior to and after MCAO, increased the degree of focal ischemic injury ($p < 0.05$).

CONCLUSIONS

Differential display is one of the most flexible and comprehensive methods available for the detection of differentially expressed genes in cells and tissues. Since its initial description, this technique has been established in many laboratories and successfully applied for the identification of genes in *in vitro* and *in vivo* systems. Similarly, other methods, such as subtractive library screening, differential hybridization, SAGE, and RDA, have also been successfully used for novel gene discovery. The

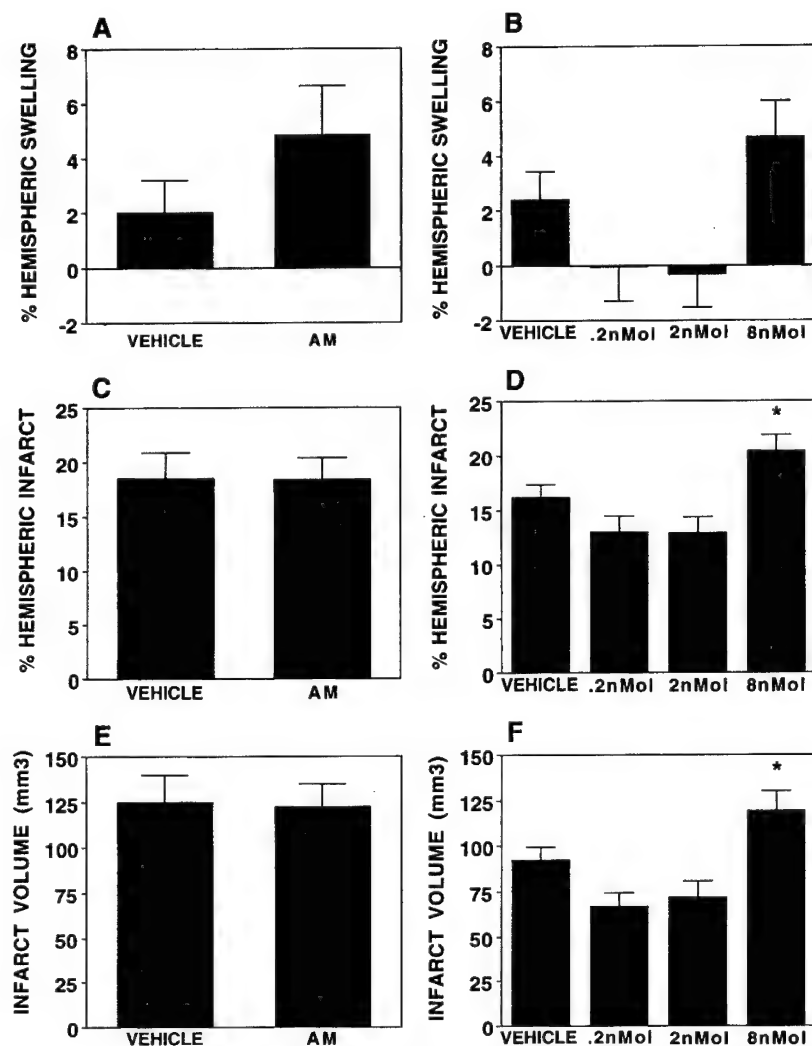


Figure 26.4 The effect of intravenous (A, C and E) and lateral ventricular (B, D and F) administration of AM are determined on percent hemispheric swelling (A and B), percent hemispheric infarct (C and D), and infarct volume (E and F) following MCAO. Intravenous AM ($n=6$) (continuously at $1\mu\text{g/kg/min}$ beginning at 1 h pre- and for 4 h post-MCAO) did not affect focal ischemic injury compared to vehicle ($n=6$) (A, C and E). Ventricular 8 nM AM ($n=13$) $5\mu\text{l}$ administrated at 1 h pre- and 6 h post-MCAO significantly increased focal ischemic injury but 0.2 nM ($n=9$) and 2 nM ($n=10$) did not compared to vehicle ($n=13$) (B, D and F; $p<0.05$). A one-way ANOVA followed by Fishers protected LSD post-hoc t -test was carried out.

application of these techniques will no doubt facilitate the discovery of novel therapeutic targets and help increase the understanding of the molecular mechanisms of disease. The same strategies are also feasible for novel gene discovery in brain under stress conditions. However, this is the first of many steps required in the discovery of a novel pharmacological target, especially since the function of the novel gene is likely to be unknown. Therefore, further actions should be taken to characterize the functions of the differentially expressed gene, including isolation of the full length cDNA, expression of the gene product for functional studies, and target validation for the importance of this gene in disease process.

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Dear Sir,

**Subject: ONR Support for
40th OHOLO Conference
on**

**New Frontiers in Stress Research:
Modulation of Brain Function**

Following ONR's generous support of the above conference, which took place in Zichron Yaakov, Israel, March 1996, we are pleased to enclose herewith a copy of the proceedings entitled: *New Frontiers in Stress Research – Modulation of Brain Function*.

Thanking you once again for all your kind assistance.

Sincerely yours,

P.R. A. D.
The Editors